

COMPLEMENT COMPONENTS CR2, FACTOR H AND I
GENETIC POLYMORPHISM AND
IMMUNOREGULATORY FUNCTION

CENTRE FOR NEWFOUNDLAND STUDIES

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MING ZHOU



Complement Components CR2, Factor H and I

Genetic Polymorphism and Immunoregulatory Function

By

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A thesis submitted to the School of Graduate studies

in partial fulfillment of the requirements of

the degree of Doctor of Philosophy

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ABSTRACT

The Regulators of Complement Activation gene cluster on chromosome 1 includes genes for regulatory proteins which interact with complement component C3. The general aim of this project was to explore genetic variation and/or immunological function of certain of these proteins.

One specific aim was to assess factor H, factor I and complement receptor 2 (CR2) genetic polymorphisms. An immunodetection system was developed to identify factor H variants separated by isoelectric focusing. The method is economical and suitable for population surveys. Allele frequencies estimated from 129 serum samples are *FH*1* 0.597 and *FH*2* 0.403. No association was observed between factor H types and rheumatoid arthritis. A new variant, *IF*B1*, was found when 288 Caucasians were typed for Factor I. The results from this survey also indicated that *IF*A* is present at a much lower frequency in Caucasians than in Asiatics. SDS-PAGE followed by immunoblotting was used to define a new, 75 kDa variant (CR2 L) of CR2 (CR2 H). Six CR2 L heterozygotes and one homozygote were observed in 63 unrelated samples and segregation of CR2 L was demonstrated by a family study. Flow cytometry analysis of the binding of EBV and various anti-CR2 monoclonal antibodies to a CR2 L homozygous cell line did not

clarify the molecular basis for the reduced size of CR2 L.

A second specific objective was to explore in vivo, using a mouse model, the regulatory role of CR2 in the immune response. Pretreatment with anti-CR2 significantly depressed the primary response (mainly IgM) to a T-dependent antigen and impaired the generation of immunological memory, but did not eradicate an already established immunological memory. The suppressive effect was different from that initiated by cobra venom factor pretreatment (C3 depletion) where only the primary IgG response was affected. Antigen complexed with anti-CR2 is physiologically analogous to C3d-Ag-Ab. Ag-anti-CR2 complexes evoked an in vivo secondary response at a 100-fold lower Ag dose than Ag alone. Taken together, these results suggest, but do not prove, that (i) when stimulated by a single signal from CR2, B cells can become anergic and (ii) CR2 can enhance the secondary response either by signalling via CR2 complexed to IgM or CD19, or by localizing Ag-Ab complexes on follicular dendritic cells.

KEY WORDS: Factor H Factor I CR2 Polymorphism
Immune response

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LIST OF ABBREVIATIONS

Ab	=	antibody
Ag	=	antigen
Ala	=	alanine
Arg	=	arginine
bp	=	base pair
°C	=	Celsius
C1-INH	=	C1 inhibitor
C4BP	=	C4b-binding protein
CD	=	cluster of differentiation
cM	=	centimorgan
cm	=	centimeter
CNBr	=	cyanogen bromide
CO ₂	=	carbon dioxide
CPM	=	counts per minute
CR	=	complement receptor(s)
CR1	=	complement receptor type I
CR2	=	complement receptor type II
sCR2	=	soluble CR2
CR3	=	complement receptor type III
CR4	=	complement receptor type IV
CVF	=	cobra venom factor
Cys	=	cysteine
DAF	=	decay accelerating factor
d.f.	=	degree of freedom
DNA	=	deoxyribonucleic acid
cdNA	=	complementary DNA
E	=	erythrocyte
-EAREVY-	=	-Glu-Ala-Arg-Glu-Val-Tyr-
EBV	=	Epstein-Barr virus
EDTA	=	diethylenetriamine tetraacetic acid
ELISA	=	enzyme-linked immunosorbent assay
FCS	=	fetal calf serum
FDC	=	follicular dendritic cells
FITC	=	fluorescein isothiocyanate
GC	=	germinal centre
Glu	=	glutamic acid
Gln	=	glutamine
gp	=	glycoprotein
h	=	hour(s)
HIV	=	human immunodeficiency virus
HRBC	=	horse erythrocytes
HRF	=	homologous restriction factor
¹²⁵ I	=	iodine-125
IFA	=	incomplete Freund's adjuvant
Ig	=	immunoglobulin
IL	=	interleukin

sIg	=	surface immunoglobulin
INF	=	interferon
kb	=	kilobase
kDa	=	kilodalton
KLH	=	keyhole limpet hemocyanin
Lys	=	lysine
M	=	molar
MAC	=	membrane attack complex
MCP	=	membrane cofactor protein
MCR	=	murine complement receptor
rMCR	=	recombinant murine complement receptor
MIRL	=	membrane inhibitor of reactive lysis
min	=	minute
ml	=	milliliter
mM	=	micromolar
m.w.	=	molecular weight
No.	=	number(s)
ng	=	nanogram
nm	=	nanometer
NP-40	=	Nonidet P-40
PBS	=	phosphate-buffered saline
pI	=	pH value of isoelectric point
PFGE	=	pulse field gel electrophoresis
PMN	=	polymorphonuclear leukocytes
RCA	=	Regulators of Complement Activation
RFLP(s)	=	restriction fragment length polymorphism(s)
RNA	=	ribonucleic acid
mRNA	=	message RNA
RPMI	=	Rosewell Park Memorial Institute
SCR(s)	=	short consensus repeat(s)
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	serine
SRBC	=	sheep erythrocytes
Thr	=	threonine
-TSQK-	=	-Thr-Ser-Gln-Lys-
Tris	=	Trishydroxymethylaminomethane
Tyr	=	tyrosine
ug	=	microgram
Val	=	valine
vs	=	versus
YAC	=	yeast artificial chromosome

CHAPTER 1

INTRODUCTION

1. History of complement research

Study of the complement system began in the late 19th century when scientific debate was focused on the mechanisms by which the body defends itself against microorganisms. At that time there were two distinct schools of thought: "cellular immunity" versus "humoral immunity". A group of humoralist giants, including Buchner, Ehrlich and Bordet, demonstrated that bacteriolysis required two factors in the bloodstream, a heat-stable factor (antibody) and a heat-labile factor (called alexin and later designated "complement"). Over the next two decades, accumulated evidence provided by the pioneering work of Ferrata, Coca, Whitehead and others indicated that complement was a complex rather than a single substance. This complexity of the complement system was not clearly elucidated until the 1940s when the development of protein purification and electrophoresis allowed individual complement components to be characterized. The nomenclature of complement components was simplified by the WHO Committee in 1968. With the help of recombinant DNA technology, a number of

studies from the past two decades have enriched and broadened our understanding of complement proteins with special emphasis on the correlation of structure and function. The discovery of cell-bound complement receptors shed new light on the biological importance of the complement system. Here, the biochemistry of complement components, activation pathways and biological functions are summarized from various reviews (Müller-Eberhard, 1988; Campbell et al., 1988; Mollnes and Lachmann, 1988; Hourcade et al., 1989; Kinoshita, 1991; Sim and Reid, 1991; Colten and Rosen, 1992).

2. The complement cascade

Complement is a major defence and clearance system that forms the principal effector arm of humoral immunity. Today, it is known that this system is a group of more than 30 different proteins, present in serum and on cell surfaces, shown in Table 1.1. Most soluble components are synthesized by the liver, although some are extrahepatically produced at local sites of injury and inflammation. Hepatocytes, macrophages, fibroblasts and epithelial cells are major sites of synthesis and secretion. The membrane-bound regulatory components, of course, are synthesized in the cells on which they are expressed. Activation of the complement system

Table 1.1. A summary of biochemical and structural features of complement components, regulatory proteins, and membrane receptors.

Components	m.w. (kDa)	Number of chains	Concen- tration (ug/ml)	Structural features
Classical pathway				
C1				
C1q	460	18	80	Collagen-like
C1r	166	2	50	Serine protease, SCR
C1s	166	2	50	Serine protease, SCR
C4	205	3	400-450	Thioester containing
C2	102	1	30	Serine protease, SCR
C3	185	2	1,300	Similar to C4
Alternative pathway				
Factor D	24	1	1	Serine Protease
Factor B	92	1	210	Serine Protein, SCR
C3	185	2	1,300	Similar to C4
Terminal proteins				
C5	190	2	70	Similar to C3/C4
C6	120	1	64	Pore-forming protein, SCR
C7	110	1	56	Similar to C6
C8	155	3	50	Pore-forming protein
C9	73	1	59	Pore-forming protein

Table 1.1. (continued)

Components	m.w. (kDa)	Number of chains	Concen- tration (ug/ml)	Structural features
Regulatory proteins				
<u>Plasma</u>				
C1-inhibitor	110	1	200	Serine protease inhibitor
C4-binding protein	570	7	250	SCR
Properdin	220	3 or 4	20	Cyclic polymer
Factor II	150	1	480	SCR
Factor I	88	2	35	Serine protease
S-protein	85	1	505	?
<u>Membrane</u>				
DAF (CD55)	70	1	N/A	SCR
MCP (CD46)	45-70	1	N/A	SCR
HRF or (C8bp)	65	1	N/A	Phosphatidyl- inositol anchor
MIRL (CD59)	20	1	N/A	Phosphatidyl- inositol anchor
Complement receptors				
CR1 (CD35)	160-250	1	N/A	SCR
CR2 (CD21)	145	1	N/A	SCR
CR3 (CD11b/CD18)	165 (α) 95 (β)	2	N/A	Leukocyte integrin
CR4 (CD11c/CD18)	150 (α) 95 (β)	2	N/A	Leukocyte integrin
C1q receptor	56	?	N/A	
C3a/C4a receptor	?	?	N/A	
C5a receptor	50	?	N/A	G protein coupling
N/A, not applicable				

initiates a sequence of biochemical reactions, each component activating the next in a cascade fashion. There are two distinct routes to activate the system: the classical and alternative pathways. Both pathways lead to the generation of C5 convertases that induce terminal assembly of the membrane attack complex (MAC) C5b-C9, as shown in Figure 1.1.

2.1. Complement activation

The classical activation pathway

The classical pathway can be initiated primarily by interaction of C1 with antigen-antibody complexes or immunoglobulin aggregates (IgG or IgM). C1 is a multimeric complex containing C1q, C1r and C1s subunits. C1q is a multichain molecule with a collagen-like stem portion and six globular heads. Multivalent binding of C1q, via its heads to specific sites in the Fc region of IgG or IgM, leads to conformational changes within C1q and C1r and results in the activation of C1r and C1s. Subsequently, activated C1s, a serine esterase, splits C4 into two fragments, C4a and C4b. By the exposed internal thioester group in the α chain, C4b can covalently bind to OH- or NH₂-groups on the target surfaces. The next stage involves the binding of C2 to C4b in the

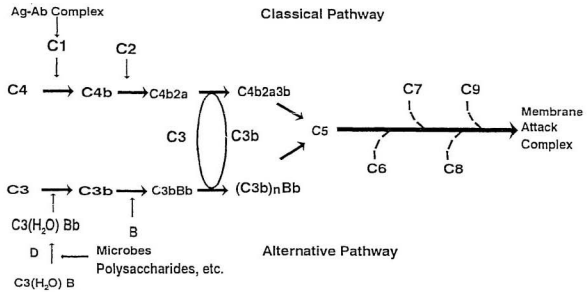


Figure 1.1. Complement System Activation

presence of magnesium ions. Following cleavage by activated C1s, C2a, a large fragment, remains associated with C4b to form a C4b2a complex, which is the classical-pathway C3 convertase. C2a in the complex cleaves C3, releasing an N-terminal fragment C3a. The remaining portion of C3, the C3b fragment, undergoes a conformational change and binds to the target surfaces in the vicinity of the C4b2a complex via an ester or amide bond. Thus, the target-bound C4b2a3b complex, the classical pathway C5 convertase, is formed.

The alternative activation pathway

The initial interaction which triggers the alternative pathway is not understood as well as that for the classical pathway. The alternative pathway activation does not depend upon antibody-antigen complexes; rather, a wide range of non-immunoglobulin activators such as fungi, yeast, lipopolysaccharides, can mediate activation. It relies on the continuous activation of C3 by a low-level hydrolysis in the fluid phase. This results in the formation of $C3(H_2O)$ capable of binding factor B, and in the presence of factor D, in the formation of the alternative pathway C3 convertase (C3bBb), which functions the same way as the C4b2a of the classical pathway to produce more C3b fragment. Next, C3b is deposited

on the target surfaces, and eventually becomes the C5 convertase (C3bBbC3b) of the alternative pathway. Compared to the classical pathway, C3 convertase (C3bBb) initiates the activation of more and more C3 by a positive feedback cycle. Thus, the alternative pathway of complement activation provides a quick, nonspecific natural defence system.

Activation of C5 and Assembly of the membrane attack complex (MAC)

C5 can be activated by either the classical (C4b2a3b) or the alternative (C3bBbC3b) pathway. After cleavage of C5 by the C2a or Bb subunit of the C5 convertases, the smaller fragment, C5a, is released into the fluid phase and the larger one, C5b, remains attached to the target surfaces and expresses transient binding sites for C6. The C5b-6 complex is subsequently stabilized by binding of C7, C8 and C9. It has been widely accepted that C9 in the MAC is present in multiple copies from 12 to 18. Although the complex C5b-8 is lytic, incorporation of C9 to the complex greatly enhances membrane perturbation and increases the size of the transmembrane pore, giving a higher efficiency of lysis.

2.2. Control of complement activation

Like any cascade system with a rapid and amplified reaction, the complement cascade must be strictly controlled, otherwise a vast amount of active intermediates from continuous activation can cause cell damage and even death. At several stages the control of complement activation is ensured by two different mechanisms: natural decay processes and active regulation. Natural decay processes occur in two ways: First, in the absence of a substrate, an active binding site will usually be inactivated over a period of milliseconds. Second, the proteins constituting a convertase will spontaneously dissociate from each other, a process that usually takes minutes. Active regulation, performed by inhibitor/regulatory proteins, also occurs in different ways. Spontaneous dissociation of a convertase can be facilitated by a regulatory protein. Alternatively, the individual proteins can be inactivated, either by changing the structure of the protein, or by degrading it into two or more pieces.

C1 level:

Control at the stage of C1 activation is achieved by C1 inhibitor, a plasma serine esterase. It not only restricts the

half-life of activated C1 but also dissociates C1r and C1s from C1q. Inhibition of activated C1 involves the covalent binding of C1-INH to the catalytic sites on C1r and C1s, followed by dissociation of these molecules from C1q. C1 inhibitor is also a potent inhibitor of other serine esterases such as plasma kallikrein, factor XIa, factor XIIa and plasmin. People with genetic deficiency of C1 inhibitor suffer from hereditary angioedema.

C3-convertases:

Several proteins are involved in control at the stage of assembly and decay of the C3-convertases (C4b2a in the classical pathway and C3bBb in the alternative pathway). Characterization of these regulatory proteins is shown in Table 1.2.

Dissociation of C2a from C4b is accelerated by C4b-binding protein (C4BP) which competes with C2a for the binding sites on C4b. C4BP then acts as a cofactor to render C4b susceptible to cleavage by the serine protease factor I. Similarly, the dissociation of Bb from C3b is accelerated by factor H, which also acts as a cofactor for cleavage of C3b by factor I. Properdin, present in the form of a mixture of cyclic polymers

Table 1.2. Proteins involved in the control and regulation of C3 convertases

Proteins	Ligands	Enzyme activity	Decay acceleration	Cofactor activity	Main Sources
Factor I	C3b/C4b	+			Plasma
Factor H	C3b	-	+	+	Plasma
C4BP	C4b	-	+	+	Plasma
Properdin	C3bBb	-	Stabilizes C3/C5 convertases		Plasma
CR1	C3b/C4b iC3b	-	+	+	E, PMN B, some T, FDC, Platelet Monos
CR2	C3d C3dg	-	-	+	B, FDC a few T
DAF	C4b2a C3bBb	-	+	-	E All PBL
MCP	C3b	-	-	+	All PBL

Abbreviations: E, erythrocytes; PMN, polymorphonuclear leukocytes; B, B lymphocytes; T, T lymphocytes; FDC, follicular dendritic cells; Monos, monocytes; PBL, peripheral blood leukocytes.

of a 56 kDa polypeptide, is another regulator in the alternative pathway. It has the opposite effect of factor H, i.e. it stabilizes the alternative C3 convertases, thus preventing the inactivation of C3bBb by factor I and H. Factor I is a regulator enzyme involved in the cleavage of the α' -chains of activation products (C3b and C4b) of complement C3 and C4. These specific cleavage reactions cause C3b and C4b to lose their ability to function in the C3 convertases. Regulation of C3 convertases is also mediated by membrane-bound molecules, including decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), complement receptor type 1 (CR1, CD35) and probably complement receptor type 2 (CR2, CD21). The genes encoding these six complement proteins, CR1, CR2, DAF, MCP, C4BP and factor H are closely linked on chromosome 1 in a region now known as the regulators of complement activation gene cluster (RCA). The RCA protein group is also sometimes called the superfamily of C3b/C4b binding proteins, based on their ligand binding abilities, functional similarities and genetic linkage. The structural and functional characterization of factor I and its six cofactors will be reviewed in detail in CHAPTER 2.

C5 and the membrane attack complex (MAC):

Control of the C5b-7 complex is mediated by several plasma inhibitors. The S-protein is the most efficient and probably the major controlling factor of the lytic potential of the MAC. This glycoprotein with a single chain of 80 kDa can bind to C5b-7, forming a SC5b-7 complex. The binding of S-protein thus prevents the C5b-7 complex from binding to the cell and therefore protects bystander cells against lysis by the MAC. On the membrane, the homologous restriction factor (HRF) known as C8 binding protein (C8bp), and the membrane inhibitor of reactive lysis (MIRL, CD59), have the capacity to regulate the assembly of the MAC, by inhibiting the binding and polymerization of C9. It is known that human complement is more efficient at lysing erythrocytes of other species than at lysing homologous erythrocytes. This phenomenon has been termed homologous species restriction of complement-mediated hemolysis. This may also be the case in other species. HRF is a 65 kDa protein and is localized on erythrocyte and leukocyte membranes. Whereas DAF, MCP, CR1 and probably CR2 regulate activation on the membrane at the level of C3 convertases, HRF serves to control the final assembly of the terminal complement complex to protect autologous cells against complement-mediated lysis. It is able to inhibit the channel-

forming function of both polyC9 and C5b-8. There is an abnormally high amount of hemolysis in patients with paroxysmal nocturnal hemoglobinuria (PNH), a disease in which cell lacks HRF. The complete functional role of HRF is yet to be defined.

Membrane Inhibitor of Reactive Lysis (MIRL) is an 18 kDa glycosyl phosphatidylinositol-anchored membrane protein that inhibits complement-mediated lysis by binding to C5b-8 and inhibiting C9 multiplicity. It also appears to modulate the activity of the alternative pathway C3 convertases, although the mechanism of regulation has not been elucidated.

3. The Biological Role of complement activation

The complement system is one of the most important humoral systems mediating many activities that contribute to inflammation and host defence, even in the preimmune phase when specific antibody and lymphocytes are not available. Activation of the complement system results in the production of molecules with potent biological activities. The major biological functions of these activation fragments are exerted by binding to their specific receptors.

Inflammation: Activation of the complement system by an infectious agent causes the release of three anaphylatoxins, C3a, C4a and C5a, from their parent molecules. They are very similar in structure and mediate a similar set of activities but with different efficiencies (C5a >> C3a >> C4a). The anaphylatoxins induce smooth muscle contraction and enhance vascular permeability by binding to specific receptors and inducing the release of vasoactive amines such as histamine from mast cells and basophils and lysosomal enzyme release from granulocytes. C5a functions also as a chemotaxin, inducing the migration of leukocytes into an area of infection or tissue destruction.

Cell lysis: Complement was originally discovered by its lytic activity. Insertion of MAC into the cell membrane results in membrane disturbance and leakage of intracellular material. Complement-mediated lysis has been shown for many kinds of cells: platelets, Gram-negative bacteria, viruses possessing a lipoprotein envelope, and lymphocytes.

Opsonization and phagocytosis: Phagocytes are able to ingest and eliminate cell debris, immune complexes and foreign microorganisms only if they can recognize and distinguish them from host cells. This requires that these targets have been

marked by antibody and/or complement fragments. This process of coating targets with antibody and/or complement fragments is termed "opsonization". The phagocyte population includes macrophages, monocytes and polymorphonuclear leukocytes. Phagocytes use their Fc receptors for arrest of antibody-coated targets, and use their complement receptors for capture of targets coated by C3 fragments. The interaction of Fc receptor and antibody is sufficient to trigger the ingestion process provided the level of antibody on the target is high enough to initiate a stable binding to phagocytic cells. In the case of only a small amount of antibody on the target, active C3b and C4b fragments deposited on the target will facilitate uptake by phagocytes by enhancing the efficiency of phagocyte binding via the complement receptors.

Immune complex clearance: Interaction of antibody and antigen in vitro can result in the formation and precipitation of a large immune complex. In vivo, precipitation of immune complexes in tissues is strongly inhibited by complement. The size of the complex formed is dependent on the ratio of antigen to antibody and, once it reaches a certain size, maximum precipitation occurs. Preformed immune complexes can be solubilized by the action of the alternative pathway components C3, B and D, resulting in both antigen and antibody

in the complex becoming coated with C3b. Association of IgG or IgM antibodies with antigen initiates the classical pathway component C1 activation. Binding of C1 to the immune complex also causes inhibition of precipitation, followed by C3b coating of the complex. Once immune complexes are coated with C3b, they can be recognized by the CR1 receptor on red blood cells and eliminated from the circulation by monocytes in the bloodstream or by macrophages in liver and spleen.

Immune regulation: Although the complement system has been studied for over a century, possible roles in the immune response for the various complement components and fragments were not formulated until the 1970's. This topic will be addressed in detail in CHAPTER 3.

CHAPTER 2

FACTOR I, ITS COFACTORS AND RELATED PROTEINS

2.1. Factor I

2.1.1. Biochemical properties

Factor I (formerly C3b inactivator, C3bINA) is involved in the regulation of the C3 convertases of either pathway. Human factor I is synthesized in liver and in extrahepatic tissues (monocytes and endothelial cells) as a single chain precursor of 88 kDa, which is then processed to yield the active form, of two disulfide-linked chains of 50 kDa and 38 kDa. Factor I is found in plasma at a concentration of about 35 ug/ml (Pangburn et al., 1977). It has been suspected for some time that factor I might belong to the serine protease family. Definitive evidence was obtained from amino acid sequence studies on the 38 kDa light chain which showed the presence of the characteristic set of residues found in the active sites of other serine proteases such as chymotrypsin and trypsin (Yuan et al., 1986).

2.1.2. Genetics

The known amino acid sequence of the 38 kDa light chain of factor I was used to generate oligonucleotide probes which allowed the isolation, from a liver cDNA library, of a clone containing the whole of the coding region (Catterall et al., 1987). The mRNA size in liver is 2.4 kb and the cloned DNA has 1750 bp encoding the single polypeptide chain of 565 amino acids. The sequence includes a putative leader peptide of 18 residues, a cysteine rich (29 Cys residues) heavy chain of 317 residues and a catalytic light chain of 240 residues. Six potential N-linked glycosylation sites are also seen. Activation of the precursor factor I probably involves the limited proteolysis and trimming of a highly basic peptide sequence (-Arg-Arg-Lys-Arg-) located at the C terminus of the heavy chain. The gene for factor I has been mapped to human chromosome 4, at q25, by the somatic cell hybrid method (Goldberger et al., 1987; Shiang et al., 1989).

A number of cases of complete deficiency of factor I have been reported (Rasmussen et al., 1988). The RFLPs of factor I have been identified in a study of several factor I-deficient kindreds (Kolble et al., 1989), but little is currently known about the mutant genes. Inherited deficiency of factor I is

usually associated with increased susceptibility to infection by pyogens such as pneumococci, *Hemophilus influenza*, and meningococci. Some patients exhibit circulating immune complexes and/or recurrent urticaria. As would be expected from the regulatory role of factor I, affected individuals have little or no native C3 in their plasma due to the uncontrolled formation of the C3bBb complex which splits C3. Infusion of factor I in vivo restores the serum concentrations of the complement proteins (C3, factor B, etc.) to normal values in the patients (Rasmussen et al., 1988).

Genetic polymorphism of factor I was first found by Nakamura et al. (1985) in the Japanese population. Using polyacrylamide gel isoelectric focusing electrophoresis of neuraminidase-treated EDTA plasma samples followed by western blotting, 435 unrelated individuals and some families living in the Tokyo area were typed. Three different phenotypes controlled by two alleles, with frequencies of *IF*A* (acidic) 0.1069 and *IF*B* (basic) 0.8931, of a single locus were found. One year later, Nishimukai et al. (1986) published the second report of Factor I polymorphism in the Japanese population and described a modified method of typing. The gene frequencies of the two alleles calculated from this study in the western part of Japan were similar to those published by Nakamura et al.

The two allelic variants of factor I differ by charge and size homogeneity has been verified by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. There has been no DNA sequence information from individuals with different phenotypes to account for the charge polymorphism. Nor are there any functional differences between the two forms. Factor I polymorphism provides a new marker for human genetics, anthropologic studies, and forensic science. Further investigations may provide useful information about the deficiency of factor I.

2.1.3. Immunological function

The third component of complement, C3, consists of two polypeptide chains (a 110 kDa α -chain and 75 kDa β -chain) linked by one disulfide bond and by noncovalent forces (Bokisch et al., 1975; Janatova, 1980), as shown in Fig. 2.1. It contains two N-linked carbohydrate moieties at residues 63 and 917, with 8-9 and 5-6 mannose residues, respectively. During complement activation C3b is produced by proteolytic cleavage of the C3 α -chain by the classical (C4b2a) or alternative (C3bBb) pathway convertases with release of the amino terminal C3a peptide (Reid and Porter, 1981). The nascent C3b has a labile binding site and binds covalently to

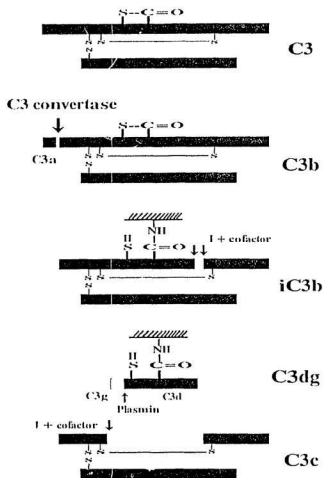


Figure 2.1. Schematic representation of C3 degradation

target molecules through an ester or amide bond to nearby hydroxyl or amino groups (Reid and Porter, 1981; Law et al., 1983). Factor I, which controls the concentration of C3b bound or in the fluid phase, is an essential part of the regulation of complement activation. The digestion of C3b by factor I proceeds in several steps and requires the presence of one of several cofactors (Reid et al., 1986; Mitomo et al., 1987). The first two cleavages of the α -chain of C3b by factor I occur between Arg-Ser residues at positions 1281-1282 and 1298-1299, liberating a 2-kDa C3f-fragment and yielding iC3b. This degradation of C3b to iC3b is accompanied by conformational changes of the molecule. An additional factor I cleavage site exists between residues 932-933 (Arg-Glu) in iC3b generating C3c and C3dg (Ross et al., 1982; Lachmann et al., 1982; Davis et al., 1984). Further proteolysis of C3dg by trypsin, elastase or plasmin generates C3d. These C3 fragments, both soluble and/or surface bound, generated during complement activation can bind specifically to several cell surface receptors (Bacherer et al., 1989).

2.2. Factor H

2.2.1. Biochemical features

Factor H is a single chain glycoprotein of 150 kDa which acts as the main soluble regulatory protein involved in the acceleration of the decay of C3bBb and as a cofactor in the factor I-mediated breakdown of C3b to iC3b. It is likely that factor H also regulates the C5 convertase since it competes with C5 for C3b (Isenman et al., 1980; DiScipio, 1981). Factor H has been described in a number of species including man. It has been found to be very similar both chemically and functionally in all species where it has been described. The concentration in plasma is 500 ug/ml in man, 230 ug/ml in mice and 130 ug/ml in rabbit (Kristensen et al., 1987). In addition to its presence in serum, factor H is also found on the cell surface of monocytes and lymphocytes, although its role here is unclear (Sim et al., 1986). A factor H membrane receptor has been identified on B cells and monocytes (Lambris and Ross., 1982).

2.2.2. Genetics

Factor H polymorphism has been described in humans and mice (Rodriguez de Cordoba and Rubinstein, 1984; Natsuume-Sakai et al., 1984). There are five allelic variants which differ by charge. This can be demonstrated using high-resolution isoelectric focusing of immunoprecipitated proteins under denaturing conditions. Each of the variants of factor H consists of one major band and four minor bands, two with slightly higher pI values and two slightly lower. The range of pI for all of the bands is 6.50-6.75. Based on the analysis of 208 unrelated Caucasians living in New York city, the gene frequencies for the factor H alleles *FH*1*, *FH*2*, *FH*3*, *FH*4* and *FH*5* are 0.685, 0.301, 0.006, 0.002 and 0.006, respectively.

Factor H variations at the cDNA level and in amino acid sequence have also been observed. The complete cDNA coding sequence has been derived from overlapping clones, and a polymorphism at base 1277 has been characterized (Day et al., 1988). In four clones there is a T at nucleotide 1277 and in two others there is a C. This T/C change represents a Tyrosine/Histidine polymorphism at position 384 in the derived amino acid sequence. Protein sequence studies on purified

factor H from pooled plasma from 12 donors confirmed the presence of both tyrosine and histidine at this position. Tyrosine and histidine were observed in a ratio of 2 : 1, respectively. This polymorphism is likely to represent a sequence difference between the two most abundant charge variants, FH 1 and FH 2. A Bgl II RFLP of factor H has also been observed.

cDNA clones have been isolated and sequenced in both humans and mice. The derived amino acid sequence indicates that the mature protein consists of 1213 residues in humans and 1216 residues in mice (Kristensen et al., 1986; Kristensen and Tack, 1986), and the two sequences are 61% homologous. Both human and mouse factor H are composed of 20 consensus repeating units of 60 amino acids. For human factor H, three different mRNA species are abundantly expressed in liver: 4.3 kb, 1.8 kb and 1.4 kb. The 4.3 kb mRNA codes for the common factor H of 150 kDa (Schwaebler et al., 1987). The sequence analysis of the 1.8 kb mRNA showed that this species is largely identical to the 5' portion of the 4.3 kb mRNA, suggesting that the 1.8 kb and the 4.3 kb mRNA arise by alternative splicing from a single structural gene (Estaller et al., 1991). The 1.4 kb mRNA is highly homologous to the 3' end of the 4.3 kb mRNA of factor H (Schwaebler et al., 1991).

The translation of the 1.8 kb mRNA is believed to produce two truncated forms of factor H, 43 kDa and 37 kDa, respectively, which have been identified in human serum by immunoblots using antisera against factor H (Estaller et al., 1991). The smaller product may be a cleaved form of the larger one. Both proteins are distinct in size from the common proteolytic products of factor H. The function and activity of these truncated forms of factor H are unclear, although they contain a sequence corresponding to the C3b-binding site. The product of the 1.4 kb mRNA is still unknown.

The chromosomal location of factor H has been determined in both humans (Hing et al., 1988) and mice (D'Eustachio et al., 1987). The human factor H gene is within the cluster of genes of RCA on the long arm of chromosome 1 (Rodriguez de Cordoba et al., 1985). Linkage analysis has suggested that the human factor H gene is approximately 6.9 cM from the rest of the genes of RCA (Rodriguez de Cordoba and Rubinstein, 1987), and gene mapping by pulse-field gel electrophoresis has confirmed this finding (Rey-Campos et al., 1988).

A number of patients have been reported who are homozygous or heterozygous deficient in factor H, and the defect at the DNA level has been identified (Brai et al., 1988;). Clinical

manifestations are quite variable. Some patients appear symptom free while others have recurrent pyogenic infections (Neisserial meningitis) or membranoproliferative glomerulonephritis (Nielsen et al., 1989; Day, 1986). The reason for these differences in clinical expression between the two deficiencies (homozygous vs heterozygous) is unclear, since both deficiencies result in increased complement activation and cleavage of C3.

2.2.3. Immunological functions

Factor H is the most abundant cofactor protein in the control of the alternative pathway of complement activation. First, it can compete with factor B for C3b binding (DiScipio et al., 1981). Second, factor H can disassemble C3 convertase by facilitating dissociation of Bb from C3b (Whaley and Ruddy, 1976). Third, factor H can inhibit C5 convertase by competing with C5 for C3b binding (DiScipio et al., 1981). Fourth, factor H can act as a cofactor for factor I to specifically cleave C3b, thereby inactivating it (Whaley and Ruddy, 1976; Pangburn et al., 1977).

Several reports have identified different regions of C3b that are involved in factor H binding, suggesting that factor

H and C3b interact via multiple sites. One of the factor H binding sites has been localized within the C3d fragment by using anti-factor H (human) anti-idiotypic antibodies and synthetic peptides. The binding site appears to be discontinuous spanning residues 1187-1249 of C3 (Lambris et al., 1988). Another site in C3b which mediates factor H binding has been localized to the N-terminal 40 amino acids of the α' chain, based on the inhibition of factor H binding to C3b by anti-C3c or anti-C3d polyclonal antibodies and by synthetic peptides. This domain of C3b (residues 727-768 of the N-terminus of the α' chain) has also been identified as a site of interaction for CR1 and factor B (Becherer and Lambris, 1988; Ganu and Müller-Eberhard, 1985).

The binding site on factor H for C3b has been localized by the use of monoclonal anti-factor H antibodies and proteolytic cleavage fragments of factor H (Alsenz et al., 1985). The trypsin-cleaved 38 kDa fragment at the N-terminus of factor H showed both the C3b-binding activity and the cofactor activity. Physical and electron microscopic studies indicated that factor H has an elongated shape which, as for C4BP, may be a consequence of the presence of 20 homologous short repeats of 60 amino acids.

2.3. C4b-binding Protein (C4BP)

2.3.1. Biochemical features

C4BP is a carbohydrate-rich plasma protein functioning as a regulator of the classical pathway of the complement system. It is the largest of the complement proteins being approximately 570,000 daltons. The concentration of C4BP in plasma is about 280 ug/ml in humans (Scharfstein et al., 1978; Fujita et al., 1978). C4BP is synthesized in the liver and its synthesis and secretion by hepatoma cells is enhanced by interleukin-6 and tumor necrosis factor, suggesting that C4BP is an acute phase protein (Hessing, 1991). Human C4BP is composed of eight chains, seven identical 71 kDa polypeptides (α chain) and one 45 kDa polypeptide (β chain). Each α chain contains 549 amino acid residues (Chung et al., 1985; Lintin and Reid, 1986). The N-terminal 491 residues can be divided into eight repeating, but not identical, homology regions of 60 amino acids of the RCA type, as described earlier (p 25). The β chain consists of 235 amino acids and the calculated molecular mass is found to be 26.4 kDa, which is in contrast with the estimated mass of 45 kDa derived from gel electrophoresis (Hillarp and Dahlbäck, 1990). The difference is caused by glycosylation of the mature molecule. The β chain

has three SCRs. The two cysteines in the C-terminal regions of both α - and β -chains are thought to be involved in the interchain disulfide bridges. Electron microscopic studies of C4BP have suggested that C4BP has a spider-like structure with seven elongated tentacles (α chain) extending from a central, ring-like core (β chain) (Dahlbäck et al., 1983; Dahlbäck and Müller-Eberhard, 1984). X-ray and neutron scattering studies suggest that C4BP in solution resembles a more rigid bundle of seven arms in contrast to the splayed-out images seen in electron micrographs (Perkins et al., 1986). The binding sites for C4b are located at the peripheral half of each α -chain (Dahlbäck et al., 1983; Fujita et al., 1985) and it has been reported that up to 4 molecules of C4b can bind to one C4BP molecule (Scharfstein et al., 1978).

C4BP appears in two forms in plasma, as free protein and in a noncovalent complex with the vitamin K-dependent coagulation protein, protein S (Dahlbäck, 1983). When C4BP binds to protein S, it inhibits its anticoagulant activity. C4BP contains one single binding site for protein S on the central core, which is distinct from the multiple binding sites for C4b (Hillarp and Dahlbäck, 1987; Suzuki and Nishioka, 1988). So far, no clear physiological function for the C4BP-protein S complex has been found.

2.3.2. Genetics

The human C4BP gene spans about 28 kb and has been mapped to chromosome 1, band 1q32, with other genes of the RCA group (Lintin and Reid, 1986; Hing et al., 1988; Rey-Campos et al., 1988; Carroll et al., 1988). The genes for both the α and the β chains are closely linked in a head-to-tail arrangement (Pardo-Manuel et al., 1990). Since the mRNA for C4BP is 2.5 kb long, a large portion of the gene must be composed of intron sequences. Human C4BP is polymorphic. In a sample of 516 unrelated Caucasian individuals, three genetic variants of C4BP have been characterized by isoelectric focusing. The gene frequencies of the three alleles are *C4BP*1* 0.986, *C4BP*2* 0.010 and *C4BP*3* 0.004, respectively (Rodriguez de Cordoba and Rubinstein, 1987). A Bgl II RFLP has been detected within the human C4BP gene but since it was present in 50% of the individuals tested it is unlikely to show any correlation with the two common variants of protein polymorphism (Lintin and Reid, 1986). No other RFLPs have been detected using other restriction enzymes. However, a number of differences between the derived protein sequence from the cDNA of an individual and the protein sequence of C4BP isolated from pooled human plasma have been found, suggesting that the protein may have more variants not yet detected by electrophoretic techniques

and RFLP analysis at the protein and DNA levels, respectively (Chung et al., 1985).

There is some documentation of changes in C4BP level in diseases (Hessing, 1991). A moderate increase of C4BP levels was observed in patients with acute pneumonia. Barnum and Dahlbäck (1990) found that C4BP is elevated in systemic lupus erythematosus. So far only one case of genetic deficiency of human C4BP has been described in a patient with atypical Behcet's disease (Trapp et al., 1987).

2.3.3. Immunological functions

C4BP regulates the activation of the classical pathway of the complement system. It is able to bind to C4b displacing C2a in the C3 convertase, C4b2a, and in addition it functions as a cofactor for factor I in the proteolytic degradation of C4b. The mechanism by which the binding of C4b to C4BP turns C4b into a substrate for factor I is unknown, but presumably it involves a conformational change in C4b. Limited digestions of human C4BP with chymotrypsin, trypsin and pepsin have indicated that the cofactor activity for factor I is located in the region of residues 177-322 and that region 332-395 may be important for binding to C4b (Chung et al., 1985).

2.4. Decay Accelerating Factor (DAF, CD55)

2.4.1. Biochemical features

DAF is mainly found as a single-chain integral membrane glycoprotein of 70 kDa present on the surface of a wide range of cell types: erythrocytes, all leukocytes, platelets, epithelial cells, and connective tissue (Kinoshita et al., 1985; Nicholson-Weller et al., 1985). Leukocyte DAF has a higher molecular mass than erythrocyte DAF, and biosynthetic studies suggest that this is due to increased glycosylation (Lublin et al., 1986). There is a soluble form present at low concentrations in extracellular fluids and tissue culture supernatants (Medof et al., 1987a).

Alternate forms of the membrane DAF molecule have also been described. A larger variant, designated DAF-2, was detected on erythrocytes by Western blotting (Kinoshita et al., 1987). DAF-2 possesses a molecular weight of 140,000, raising the possibility that it is a dimer of DAF. DAF-2 represents less than 10% of the total membrane DAF. The structure of DAF-2 remains unexplained.

The primary structure was deduced from cloned DAF cDNA, which encodes a polypeptide of 347 amino acid residues (Medof et al., 1987b; Caras et al., 1987). Beginning at the N-terminus, the protein possesses four short consensus repeats (SCRs) followed by a Ser and Thr-rich region and a hydrophobic transmembrane anchor sequence of 23 C-terminal residues.

2.4.2. Genetics

The DAF gene was initially cloned from cDNA libraries from Hela cells and HL-60 cells using oligonucleotide probes based on the NH₂ terminus DAF sequence. Linkage studies of RFLPs of genomic DNA, somatic cell hybrids, and in situ hybridization localized the DAF gene to human chromosome 1, band q32; it is part of the RCA gene cluster (Lublin et al., 1987; Rey-Campos et al., 1987). It is now known that the DAF gene is composed of 11 exons spread over 35 kb of genomic DNA and there are two species of mRNA products, one probably derived from the other by a splicing event that induced a coding frame shift near the C-terminus (Caras et al., 1987). The spliced mRNA, which accounts for 90% of DAF mRNA, encodes membrane DAF, and the unspliced mRNA produces secreted DAF. The DAF gene is a single copy (Stafford et al., 1988) and genetic polymorphism of DAF has been identified at the DNA level by RFLP analysis. Three

RFLPs have been defined so far, two for the enzyme Hind III and one for Bam HI (Stafford et al., 1988; Post et al., 1990). Serological polymorphism was also described by analysis of the Cromer-related human blood group antigens (Cr^a and Tc^a) on the DAF molecule (Telen et al., 1988).

2.4.3. Immunological functions

DAF protects cells from autologous complement damage by inhibiting the formation of C3 and C5 convertases of the classical or alternative pathways. It also accelerates the spontaneous decay of the preformed C3 convertases. The precise mechanism underlying the interference with the C3 convertases, and the specific binding sites for DAF on C3b or C4b are still unclear. Furthermore, DAF has no cofactor activity for the cleavage of C3b or C4b by factor I (Pangburn et al., 1983). This is in contrast to the other membrane (CR1, CR2 and MCP) and fluid phase (factor H and C4BP) regulators of the C3 convertases which do act as cofactors for factor I.

2.5. Membrane Cofactor Protein (MCP, CD46):

2.5.1. Biochemical features

Membrane cofactor protein was initially termed gp 45-70, to reflect its electrophoretic mobility on SDS-PAGE (Cole et al., 1985). Except for erythrocytes, MCP has been found on nearly every cell and tissue examined: peripheral blood leukocytes; platelets; sperm; trophoblast; and cells of fibroblast, epithelial, and endothelial lineages, including malignant cell lines such as Hela (Cole et al., 1985; Seya et al., 1986; Yu et al., 1986; Seya et al., 1988; McNearney et al., 1989). Peripheral blood leukocyte populations express 5,000-15,000 copies of MCP/cell while hematopoietic lines express 30,000-70,000 MCP/cell (Seya et al., 1990; Cho et al., 1991). The protein has been purified, multiple cDNA cloned and sequenced. It is composed of four short consensus repeats (SCRs) and is a member of the RCA family. An unusual feature of MCP of human peripheral blood mononuclear cells and platelets is the presence of two heterogeneous protein species of 59 - 68 kDa and 50 - 58 kDa (Cole et al., 1985). Prior to molecular studies, MCP was characterized biochemically in order to provide an explanation for the two species. It was found that the shift in molecular weight could be accounted for in part

by the variability in precursor forms, in carbohydrate unit compositions and in glycosylation (Stern et al., 1986; Ballard et al., 1988).

2.5.2. Genetics

The MCP gene is situated on the long arm of chromosome 1 at q32 within the RCA cluster (Lublin et al., 1988; Bora et al., 1989). It consists of 14 exons and 13 introns and has a minimum length of 43 kb (Post et al., 1991). At the protein level, 72 unrelated individuals have been evaluated for MCP phenotype on peripheral blood cells (Ballard et al., 1987). Three stable patterns of expression were noted: predominance of upper-form pattern in 65%; equivalence of both forms in 29%; and predominance of lower-form in 6%. Family studies further supported a two-allelic codominantly inherited system regulating expression of MCP. It has also been determined that the phenotypic pattern of MCP is the same on a given individual's mononuclear cells (B cells, T cells, and monocytes) and platelets, and that the pattern is stable over time (Ballard et al., 1987; Yu et al., 1986). More recently, a Hind III restriction fragment length polymorphism (RFLP) was found that correlated with this pattern of expression (Bora et

al., 1991). This particular polymorphic site was traced to an intron between exons 1 and 2.

No functional differences between the two major forms are known, and no MCP deficiency has so far been described.

2.5.3. Immunological functions

The wide tissue distribution of MCP suggests that it plays an important role. Like other RCA proteins, the C3b and C4b binding sites on the MCP molecule would be expected to be present within its four SCRs. By deleting one SCR and then expressing the mutant protein in COS cells, Adams et al. (1991) have demonstrated that MCP SCR-3 and SCR-4 are critical for C3b binding while SCR-2, -3, and -4 are necessary for C4b binding. These and other data suggest that the C3b and C4b binding sites are distinct and that SCR-2 contributes more to C4b than to C3b binding. On the other hand, it was also found that a mutant lacking SCR-2 bound C3b but had no cofactor activity. This suggests that ligand binding and cofactor activity can be separated, and that there is a direct interaction between a region within SCR-2 and factor I.

Like CR1 and factor H, soluble MCP acts as an efficient factor I-dependent cofactor for generating iC3b from fluid-phase C3b (Liszewski and Atkinson, 1992). However, this does not lead to further degradation to C3c and C3dg. Also, unlike CR1, MCP and factor H do not mediate the cleavage by factor I of iC3b to C3c and C3dg from membrane-bound C3b. No decay-accelerating activity has been found for MCP.

2.6. Complement Receptor type I (CR1, CD35)

2.6.1. Biochemical features

There are at least four receptors for C3 fragments, CR1, CR2, CR3 and CR4 (Sim et al., 1987; Becherer et al., 1989). In tables 1 and 2 the properties and biological effects of these C3 receptors have been summarized.

Complement receptor type I (CR1) is a receptor for C3b and C4b, the major fragments of activated C3 and C4. CR1 also has binding affinity for iC3b, a cleavage product of C3b. CR1 was first isolated from human erythrocytes, and was shown to be of 205,000 to 250,000 molecular weight on SDS-PAGE. It is an integral membrane single chain glycoprotein found on a wide range of cells which includes erythrocytes,

monocytes/macrophages, neutrophils, B lymphocytes and some T lymphocytes (Fearon and Wong, 1983). Low concentrations of soluble CR1 are also present in plasma (Yoon and Fearon, 1985). The number of receptors varies among cell types, with peripheral blood neutrophils, monocytes, and B lymphocytes having 20,000-40,000 CR1/cell, T lymphocytes having one-tenth this number and erythrocytes having an average of only 100-1,000 CR1/cell (Fearon, 1980; Wilson et al., 1983).

The ontogeny of CR1 on human B lymphocytes has been studied (Tedder et al., 1983). The receptor is present on only 15% of large pre-B cells and 35-50% of small pre-B cells in the bone marrow. Although 60-80% of immature B cells in bone marrow and fetal liver and all peripheral blood mature B cells express CR1, maturation to plasma cells is associated with the loss of CR1.

2.6.2. Genetics

Although initial investigations had suggested that CR1 was a single chain polypeptide, later studies found some variation in the size of CR1 isolated from different individuals. It is now known to be a polymorphic protein with four allotypes that differ in molecular weight but do not differ in their capacity

to bind C3b/C4b. The common allotype, A, with a gene frequency of 0.82, has a molecular mass of 190 kDa while the B allotype, with a gene frequency of 0.18, appears to be 220 kDa. Rare CR1 allotypes C and D have a higher, 250 kDa and a lower, 160 kDa molecular weight, respectively (Dykman et al., 1983, 1984, 1985; Wong et al., 1983; van Dyne et al., 1987). The basis for the large difference in molecular mass between the allotypes was shown to be a protein difference rather than a difference in glycosylation, and later genetic studies showed that it was due to duplication of homologous domains (Wong et al., 1986, 1989).

The primary sequence of the A allotype of CR1 has been deduced from the cDNA sequence and includes a 41-residue signal peptide, an extracellular domain of 1930 residues, a 25 amino acid transmembrane domain, and a 43 amino acid cytoplasmic region (Klickstein et al., 1987, 1988). The extracellular domain includes thirty short consensus repeats (SCRs) similar to those present in other C3b/C4b binding complement proteins. As shown in Fig. 2.2, the 5' 28 SCRs of CR1 are organized into four tandem long homologous repeats (LHR) termed LHR-A, -B, -C and -D, 5' to 3', respectively. (Note, the LHR terminology A, B, C and D does not correlate

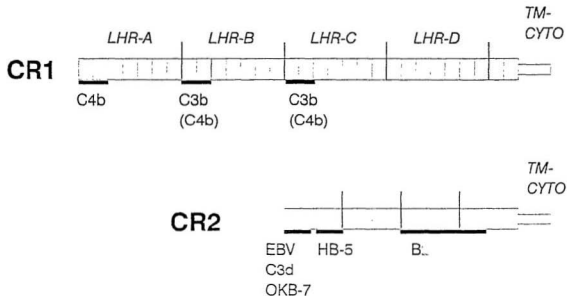


Figure 2.2. Schematic representation of human CR1 and CR2. Boxes, short consensus repeats (SCRs), some comprising long homologous repeats (LHR); —, ligand binding sites (such as C3b, C3d) or epitopes (such as HB-5). *TM-CYTO*, transmembrane and cytoplasmic domains;

with the allotypes A, B, C and D.) Each contains seven SCRs encoding about 450 amino acids, and the sequence homology among the corresponding SCRs in each LHR ranges from 60% to 99%. Distinct binding sites for C3b and C4b in LHR-A, -B and -C have been demonstrated by analysis of the formation of rosettes between the COS transfected CR1 mutants and C3b- or C4b-coated sheep erythrocytes, indicating that a LHR represents a structural and a functional unit. Two distinct ligand binding sites for C3b were found to reside in LHR-B and -C, respectively, and an additional binding site for C4b was located in LHR-A. LHR-D had no C3b or C4b binding function.

The allotypic differences in size by increments of 30 Kda may be accounted for by the variable numbers of LHRs. Direct evidence in support of this proposal was obtained by isolating and mapping overlapping phage and cosmid clones from a genomic library prepared with DNA from an individual homologous for the B allele (Wong et al., 1989). It was found that the B allele has an additional genomic segment which appears to encode an additional LHR resembling a chimera of the 3' region of LHR-A and the 5' region of LHR-B. This allele most likely arose through an homologous recombination event involving the LHR-A and -B region, and similar mechanisms of duplication or deletion by homologous recombination with unequal cross-over

may account for the other two rare CR1 allotypes. Thus, the smallest allotype C would contain 3 LHRs while the largest allotype D would contain 6 LHRs.

The CR1 polymorphism was also observed at the transcription level (Wong et al., 1986; Holers et al., 1987). The individuals expressing the various CR1 phenotypes had incremental differences of about 1.4 kb in mRNA. This difference corresponded to the size of one LHR and the 30 kDa differences seen among allotypes of CR1, further suggesting that intragenic duplication or deletion of the LHR rather than alternative mRNA splicing formed the basis for the structural polymorphism.

The number of CR1 molecules on erythrocytes can be quite different from one individual to another (Wilson et al., 1982). In addition to an allotypic variation in the size of CR1, the number of CR1 per erythrocyte (CR1/E) is also a heritable trait. A restriction fragment length polymorphism (RFLP) that correlates with either low or high numbers of CR1/E has been identified in the CR1 gene using Hind III (Rodriguez de Cordoba and Rubinstein, 1986; Wilson et al., 1986; Wong et al., 1989). Individuals may express either very low numbers of CR1/E (6.9 kb Hind III fragment), high numbers

of CR1/E (7.4 kb fragment), or have intermediate numbers of CR1/E (heterozygotes with both 6.9 and 7.4 kb fragments). Whether this genomic polymorphism has direct effects on transcription or whether it represents a marker for other factors awaits the definition of the promoter/enhancer elements in the CR1 gene and a comparative study of their relative regulatory capacities in individuals who express high or low CR1 numbers.

The human gene for CR1 is linked to the genes for other RCA proteins at band q32 on chromosome 1 (Weis et al., 1987). No complete deficiency states for CR1 have been reported but a decreased number of CR1 is found on erythrocytes, neutrophils, and B lymphocytes of patients with systemic lupus erythematosus (SLE) (Iida et al., 1982; Atkinson, 1986; Wilson et al., 1982, 1986). It was initially proposed that inheritance of low numbers of CR1/E might be a risk factor that contributed to the onset of this autoimmune disease. However, further studies suggested that the reduced CR1 levels found in patients with SLE are acquired and not inherited (Cohen et al., 1989). Finally, studies of patients with SLE and their families for the 6.9 kb RFLP associated with low CR1/E showed no correlation of the RFLP with SLE (Moldenhauer et al., 1987). Even families with a history of SLE did not

exhibit an abnormally higher incidence of the low CR1/E RFLP (Tebib et al., 1989). It is therefore likely that the CR1 reduction observed in SLE patients is an acquired characteristic (Walport and Lachmann, 1988).

2.6.3. Immunological functions

CR1 was the first membrane protein to be characterized as an inhibitor involved in complement activation. CR1 can regulate alternative pathway activation by three mechanisms: impairing uptake of factor B by C3b; displacing Bb from the C3bBb C3 convertase; and promoting the cleavage of C3b to iC3b, C3c, and C3dg by factor I. Similarly, CR1 inhibits the classical pathway by impairing uptake of C2 by C4b, displacing C2a from the C4b2a C3 convertase and from the C4b2a3b C5 convertase, and promoting the cleavage of C4b to C4c and C4d by factor I (Iida and Nussenzweig, 1981). Despite its ability to promote the decay of C3 and C5 convertases, CR1, unlike DAF and MCP, does not appear to play an essential role in the protection of erythrocytes from homologous complement attack (Medof et al., 1987). The binding site for CR1 on C3b has been localized to a domain in the N-terminus of the α' chain, spanning residues 727-768 of the C3 sequence (Becherer and Lambris, 1988). A synthetic peptide spanning this region of

the C3 α' chain, as well as the corresponding antipeptide antibody, inhibited CR1 binding to C3b and C3c. This region of C3 has also been shown to be involved in the interaction of C3b with factor H and factor B (Ganu and Müller-Eberhard, 1985; Becherer and Lambris, 1988; Becherer et al., 1989).

CR1 has important roles as a receptor. The most striking role of CR1 in vivo is to transport complement-coated immune complexes, and subsequently to enhance clearance of these immune complexes by the phagocytic system (Cornacoff et al., 1983; Schifferli et al., 1986). First, due to the greater abundance of erythrocytes as compared to leukocytes, immune complexes that enter or form in the blood bind primarily to erythrocytes. Second, despite expressing relatively fewer CR1 molecules per cell, erythrocytes are able to bind immune complexes efficiently because their CR1 are located in large clusters on the cell surface. Both soluble and particulate complement-coated immune complexes can be carried by erythrocyte CR1 to the liver and spleen, where phagocytic cells will ingest the immune complexes. This occurs in two ways: enhancement of the Fc receptor-mediated phagocytosis of particles bearing C3b and IgG, and direct phagocytosis of the C3b-bearing particle mediated by CR1 independently of Fc receptors. Additionally, the therapeutic potential of CR1 as

an inhibitor of complement-mediated inflammatory reactions is supported by a study in which soluble recombinant CR1 reduced the sizes of myocardial infarcts in a rat model of reperfusion injury (Weisman et al., 1990).

All mature B lymphocytes, a subset of T lymphocytes, and most antigen-presenting cells express CR1. The involvement of CR1, as well as other complement components in immunoregulation will be reviewed in the next chapter.

2.7. Complement receptor type II (CR2, CD21)

2.7.1. Biochemical features

The cell-surface receptor for the C3 fragments C3dg and C3d was termed complement receptor type 2, CR2. In 1983, the receptor was first clearly characterized as a discrete B-lymphocyte molecule of about 140 kDa (Iida et al., 1983). Earlier experiments also showed that Epstein-Barr virus (EBV), a human herpesvirus, could bind to CR2, and initiate the infection of B lymphocytes (Fingerth et al., 1984; Nemerow et al., 1985). A recent study further suggested that CR2 may also function as a receptor for interferon α (Delcayre et al., 1991).

The primary protein structure of CR2 has been determined through analysis of the nucleotide sequences of clones isolated from human tonsillar and B lymphoblastoid cell line (Raji) cDNA libraries (Moore et al., 1987). Four of the five clones obtained from tonsillar libraries encode a protein composed of 1032 amino acids, including a 20-amino acid signal peptide, a 954-residue extracellular domain, a 24-amino acid transmembrane region, and a 34-amino acid cytoplasmic domain. The extracellular domain is composed entirely of 15 short consensus repeats (SCRs) which are homologous to those found in other complement C3b/C4b binding proteins. One of the five clones from the tonsillar library and the single clone from Raji cells encoded a total of 16 SCRs, with an extra SCR (10a) inserted between SCRs 10 and 11 of the 15-SCR form of CR2 (Weis et al., 1988). Recent studies strongly suggested that alternative splicing is responsible for exclusion of the 10a SCR which is encoded by a single exon (Fujisaku et al., 1989; Toothaker et al., 1989). CR2 SCRs can be grouped into four "LIRs-like" domains (I-IV), based on their relative homology (Fig. 2.2).

Evidence from biosynthetic and enzymatic approaches indicate that CR2, which contains N-linked oligosaccharides, is derived from a 110-120 kDa non-glycosylated precursor with

potential sites for glycosylation in its extracellular domain (Weis and Fearon, 1985). Although the half-life of glycosylated CR2 on the cell membrane is much longer than that of non-glycosylated CR2, the oligosaccharide residues are not necessary for CR2 binding to C3d/C3dg. The cytoplasmic domain contains three serine, two threonine, and four tyrosine residues, including the sequence -TSQK-. This is the best candidate for a protein kinase C substrate (Kishimoto et al., 1985). The cytoplasmic domain of CR2 is too small to encode a tyrosine kinase, but it is possible that the sequence -EAREVY- may serve as a substrate for tyrosine phosphorylation (Hunter and Cooper, 1985).

Human CR2 has been found on mature B lymphocytes (Iida et al., 1983; Weis et al., 1984), follicular dendritic cells (Reynes et al., 1985) and epithelial cells (Young et al., 1986; Birkenbach et al., 1992), thymocytes (Tsoukas and Lambris, 1988) and some T lymphocytes (Sauvageau et al., 1990; Fischer et al., 1991). CR2 expression on various cell types or lymphoblastoid cell lines is quite different from one to another. For example, tonsillar mononuclear cells express about 17,000 CR2 per cell while peripheral mononuclear cells express 12,000 CR2 per cell; and the Burkitt B lymphoma cell line Raji expresses 24,000-63,000 receptors per cell as

compared with 8,000 receptors per cell for Molt-4, a T cell leukemia line (Fingerhuth et al., 1984).

Analysis of CR2 expression during human B cell differentiation, using monoclonal antibodies to CR2, showed that pre-B and immature B cells from fetal bone marrow and liver did not express CR2 (Tedder et al., 1984). CR2 expression is characteristic of the mature B cell, but it is lost during the differentiation from B cell to plasma cell.

A soluble form of CR2 (sCR2) has been shown to be present in easily detectable amounts in culture supernatants of B lymphoblastoid cell lines like Raji, and in normal human serum (Ling et al., 1991; Ling and Brown, 1992). The tissue source and function of sCR2 are unknown. It may represent a spontaneously shed proteolytic fragment of CR2.

2.7.2. Genetics

The CR2 gene spans 25 kb within the RCA cluster located on human chromosome 1, band q32 (Weis et al., 1987, 1988; Fujisaku et al., 1989). The fully processed mRNA is 5 kb (Weis et al., 1986). At the DNA level, CR2 polymorphisms have been identified by comparison of the DNA sequences of the published

CR2 clones from different laboratories (Moore et al., 1987; Weis et al., 1988). As described above, some of these clones have 15 SCRs while others have 16 SCRs. Moreover, both mRNA forms have been found (Toothaker et al., 1989). Thus, these data suggest that alternative use of an extra exon encoding SCR 10a can produce two forms of CR2.

Several allelic forms of CR2 have been identified on the basis of the existence of restriction fragment length polymorphisms of genomic DNA (Fujisaku et al., 1989). The Taq I RFLP involves two bands 2.55 and 2.1 kb. By utilizing more specific subclones on Southern blots of genomic DNA and cosmid clones, this polymorphic site is near the single exon encoding SCRs 1 and 2. Family studies indicate that these bands are allelic. Gene frequencies in Caucasians are 0.7 and 0.3 for the 2.55 and 2.1 kb fragments, respectively. Similarly, a Hae III RFLP has also been found. There are three bands of 1.75, 1.55 and 1.45 kb at gene frequencies of 0.02, 0.05 and 0.93, respectively, in Caucasians.

Unlike other proteins encoded by the RCA gene cluster, CR2 polymorphism at the protein level has not yet been reported.

2.7.3. Immunological functions

The functions of CR2 have been more difficult to define than those of other C3b/C4b binding proteins. It is known that CR2 is a membrane regulatory protein of complement activation, and that CR2 serves as a receptor on B cells for both C3d fragments, and EBV (Fingerth et al., 1984; Nemerow et al., 1985). It has also been implicated in the modulation of immune responses although the mechanisms by which this occurs are not well understood. Other biological roles for CR2 have recently been postulated. CR2 may act as an interferon- α receptor (Delcayre et al., 1991) and CR2 on T lymphocytes may mediate infection by human immunodeficiency virus (HIV) independently of CD4, the well-defined HIV target on T helper cells (Boyer et al., 1991, 1992; Montefiori et al., 1992). Finally, CR2 has also been identified as the ligand for the soluble CD23 (Aubry et al., 1992).

CR2 regulates the complement cascade by serving as a cofactor for factor I-mediated cleavage of membrane-bound C3b into iC3d (Kinoshita et al., 1990), and into C3c and C3dg (Mitomo et al., 1987). As this cofactor activity was blocked by CR2-specific monoclonal antibodies, it was concluded that CR2 rather than CR1 was responsible for the effect.

The binding site on C3d for CR2 was initially identified within a 8,600 dalton CNBr fragment of C3d, and then more precisely localized, by a series of overlapping synthetic peptides, to residues 1209-1236 of the C3 sequence (Lambris et al., 1985). This region is sandwiched within the discontinuous factor H binding site (Lambris et al., 1988) and may yield a plausible explanation for the factor H-like cofactor activity of CR2 (Mitomo et al., 1987). Also, this region is similar to a sequence of the gp350/220 glycoprotein of EBV at residues 21-31, suggesting that EBV may bind to CR2 via the 21-31 sequence (Nemerow et al., 1987). Synthetic peptides from this sequence of gp350/220 bind to CR2 and compete with CR2-binding peptides derived from C3d (Nemerow et al., 1989). Furthermore, the CR2 specific monoclonal antibody OKB-7 inhibits both C3d and EBV binding to CR2 (Nemerow et al., 1985), and a deletion mutant of gp350/220, missing two amino acids from the region of similarity with C3d, does not bind CR2 (Tanner et al., 1988). In contrast, data obtained using CR2-specific monoclonal antibodies and an anti-idiotypic anti-CR2 antibody indicated different binding sites on CR2 for C3d and gp350/220 (Barel et al., 1988). Recent studies have provided a conclusive answer to this question. A series of transfectants of truncated CR2 mutants were used to map the epitopes on CR2 for C3d, EBV, and CR2-specific monoclonal antibodies. The C3d,

EBV and anti-CR2 mAb OKB-7 binding sites are found in the two amino-terminal SCRs of CR2. SCRs 3-4 express an epitope for anti-CR2 mAb HB-5 while SCRs 9-14 express an epitope for anti-CR2 mAb B2 (Lowell et al., 1989; Carel et al., 1990). The intracytoplasmic domain of CR2 is not required for binding of C3d or EBV but is necessary for internalization of cross-linked C3d as well as EBV infection of cells (Carel et al., 1990).

EBV is an oncogenic herpesvirus which causes acute infectious mononucleosis, and is implicated in the pathogenesis of Burkitt's lymphoma, nasopharyngeal carcinoma (Tosato, 1987), and some autoimmune diseases such as rheumatoid arthritis (Roudier et al., 1989). It is capable of infecting and immortalizing human B lymphocytes in vitro. Experiments have shown that EBV infection is mediated by gp350/220 binding to CR2 on B cells (Tanner et al., 1987; Nemerow et al., 1987). Although CR2 can bind EBV it has not been determined whether cellular expression of CR2 is sufficient for infection. This question was addressed in part by stably transfecting murine L cells with a full-length human CR2 cDNA (Cantaloube et al., 1990). Transfectants expressed high levels of CR2 and specifically bound both C3d and EBV. Coculture of these cells with EBV led to infection of

approximately 0.5% of the cells, as assessed by staining for Epstein-Barr nuclear antigen (EBNA). The low efficiency of infection compared with human B cell lines may indicate that additional factors are involved in determining EBV tropism. With regard to virus infection, recent studies have led to the conclusion that CR2 mediates human immunodeficiency virus type I infection of a human T cell line in a CD4-independent fashion (Boyer et al., 1991, 1992; Montefiori et al., 1992). CD4 is believed to be the primary receptor for HIV on human T cells. The presence of CR2 on T cells and other target cells may thus be critical for massive infection in normal individuals, and for viral propagation in infected patients (Tosoukas and Lambris, 1993).

Surprisingly, computer analysis of the secondary structure indicated that interferon α (INF- α) also contains a sequence motif similar to the CR2-binding site of C3d (Delcayre et al., 1991). Experiments have shown that antibodies against a peptide with the CR2 binding sequence of C3d react with a peptide carrying the IFN- α CR2 binding motif (residues 92-99). This motif is conserved in most IFN- α subtypes. The IFN- α derived peptide, as well as recombinant IFN- α , inhibits C3d interaction with CR2 on Raji cells. Direct interaction of IFN- α with CR2 is inhibited by polyclonal anti-INF- α , anti-CR2 and

anti-C3d peptide antibodies as well as C3d, gp350/220 of EBV but not by interferon- γ (IFN- γ). And finally, IFN- α binding to Raji cells can be blocked by anti-CR2 monoclonal antibody HB-5 but not OKB-7. As mentioned above, HB-5 can block neither the binding of C3d nor gp350/220 to CR2. Thus, CR2 may have a distinct site for the IFN- α binding. It is likely that CR2 possesses multiple sites of interactions, one for both C3d fragments and gp350/220 of EBV, and one for INF- α . Such a model would be analogous to the CR1 interaction with C3b and C4b.

CR2 is postulated to play a functional role in B cell activation and differentiation (Cooper et al., 1988; Ahearn and Fearon, 1989). The nature of the ligand interacting with CR2 appears important since monovalent fluid-phase C3d fragments generally inhibit in vitro B cell responses while multivalent CR2 ligands have been shown to induce growth and differentiation of B cells. Our current knowledge of the regulation of immune responses by complement components including CR2 will be detailed in the next chapter.

2.8. Murine complement receptor family

The existence of mouse complement receptors that are functional homologs of human CR1 and CR2 was inferred long ago. Sheep erythrocytes bearing mouse or human C3b form rosettes with mouse spleen cells and macrophages. An immune adherence and immune complex clearance mechanism, similar to the human CR1 mechanism, exists in mice, although complement-coated complexes bind to mouse platelets rather than erythrocytes (Kinoshita et al., 1985).

Recent studies have demonstrated three C3-binding proteins in mice (Kinoshita et al., 1985; Wong and Fearon, 1985). These have been designated p190 (mouse CR1), p150 (mouse CR2), and p65. Interestingly, when mAbs were raised to the mouse p190, two of the three mAbs also recognized the mouse p150 (Kinoshita et al., 1988). These data suggested that the two mouse complement receptors are closely related to each other antigenically, and perhaps structurally. Surprisingly, despite extensive searches, no DAF or MCP genes have been discovered in mice (Krych et al., 1992).

It is now known that unlike the human system, in which CR1 and CR2 are independent gene products, the p190 is the product

of alternative splicing within the mouse CR2 gene (Kurtz et al., 1990; Molina et al., 1990). The p150 (mouse CR2) is highly homologous to human CR2 (Kurtz et al., 1989; Fingerth, 1990). These conclusions were primarily drawn from studies of recombinant transfectant cells. There are two transcripts from a common gene. One transcript encodes 15 SCRs with exon-intron junctions identical to human CR2. The second transcript encodes 21 SCRs and is generated by the addition of six extra SCRs to the first CR2 SCR. The recombinant 15 SCR form, rMCR2, has been expressed in the human erythroleukemia cell line K562. The transfected cells express a 150 kDa protein that is identical in size and function to the natural mouse CR2 (Molina et al., 1991). Cells transfected with the recombinant 21 SCR, rMCR1, yielded a 190 kDa protein that reacts with a monospecific anti-mouse CR1 mAbs as well as anti-CR2 mAbs. This 21-SCR containing rMCR1 molecule also binds C3b as well as C3dg (Molina et al., 1992). Therefore, it is believed that mouse CR1 and CR2 arise by alternative splicing from a common gene. In Fig. 2.3 is shown a schematic representation of the two receptors along with p65, a gene product apparently unique to the mouse. The p65 gene, designated *Crry*, contains five SCRs with significant sequence identity to SCRs 1-6 of MCR1.

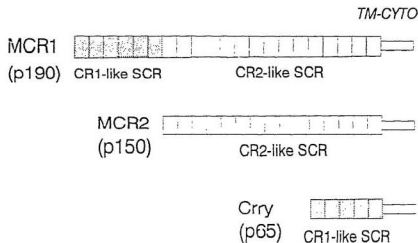


Figure 2.3. Schematic representation of mouse homologues of human complement receptors. MCR1 is the product of an alternatively spliced mRNA derived from the MCR2 gene. Crry/p65 is an intrinsic complement regulatory protein, whose five SCRs are significantly homologous to human CR1. *TM-CYTO*, transmembrane and cytoplasmic domains.

2.9. Superfamily of Short Consensus Repeats (SCRs)

As discussed earlier, the regulators of complement activation include six proteins: factor H, C4BP, DAF, MCP, CR1, and CR2. One of the common features of the six proteins is a conserved motif of approximately 60 amino acids, which is characterized by having a framework of four invariant cysteines (disulfide-bonded within each motif in the manner 1-3, 2-4) and several other highly conserved residues including proline, tryptophan, tyrosine/phenylalanine and glycine. This structural feature was designated as short consensus repeat (SCR) (Reid, 1986; Klickstein et al., 1987). Thus, the RCA proteins are related structurally as well as functionally. The first evidence that the RCA proteins might have a common evolutionary origin came from linkage studies of polymorphic variants of CR1, factor H, and C4BP (Rodriguez de Cordoba et al., 1985). CR2, DAF and MCP were then added to the list of members of the RCA family (Ray-Campos et al., 1987; Carroll et al., 1988; Bora et al., 1989). Now, genetic mapping by pulse field gel electrophoresis (PFGE) and the yeast artificial chromosome (YAC) technique has shown that the genes for CR1, CR2, DAF, MCP and C4BP are all located in band q32 of the long arm of human chromosome 1. This region of about 900 kb encodes six RCA genes and three gene-like elements (pseudogenes) in

the order (5' to 3') of C4BP- β , C4BP- α , C4BP-like, DAF, CR2, CR1, MCP-like, CR1-like and MCP (Ray-Campos et al., 1987; Carroll et al., 1988; Bora et al., 1989; Pardo-Manuel et al., 1990; Hourcade et al., 1992). The gene for factor H, though belonging to this cluster, is about 500 kb away (Fig. 2.4).

In addition to being present in the complement control proteins, SCRs are also found in C2, factor B, C1r/C1s, C6 and C7 (Hourcade et al., 1989; Reid and Day, 1989). All of these proteins can bind to C3 or C3b, or to its homologs C4 and C5. Intriguingly, a number of non-complement proteins have been shown to contain SCRs, such as human β_2 -glycoprotein, IL-2 receptor, the blood clotting factor XIIIb subunit, haptoglobin, the core protein from cartilage proteoglycan, and thyroid peroxidase. Furthermore, SCRs have been identified in a recently described family of cell adhesion proteins, which are involved in the targeting of white blood cells to appropriate sites. These proteins include the endothelial leukocyte adhesion molecule I (ELAM-1), the mouse lymph node homing receptor and the 140-kDa granule membrane protein (Reid and Day, 1989).

The C3b/C4b binding proteins are now seen as a new family derived from a common ancestral SCR-encoding genetic element.

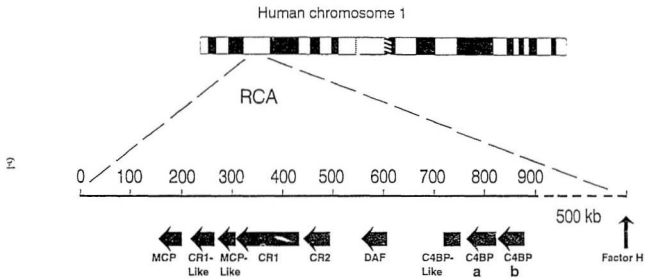


Figure 2.4. Organization of the genes within the human RCA cluster

The evolutionary mechanisms apparently involved exon split, gene duplication (intra and extra), and alternative polyadenylation (Hourcade et al., 1989).

To better understand the protein-protein recognition role of the SCR, several low-resolution structure determination techniques have been applied. These include low-angle X-ray and neutron scattering of C4BP and factor H (Parkins et al., 1986, 1991), and electron microscopy of C4BP, recombinant CR1 and recombinant CR2 (Dahlback et al., 1983; Weisman et al., 1990; Moore et al., 1989). These studies led to the proposal of a structure of " β -sheet-sandwich-type" model for the SCR. Recently, the two- and three-dimensional structure, based primarily on nuclear magnetic resonance data of one SCR, namely the 16th SCR from human factor H, has been described (Barlow et al., 1991; Norman et al., 1991). It features β -strands with interspersed turns and loops. One face of the SCR is formed by a triple-stranded β -sheet and the other face by two segments of double-stranded antiparallel β -sheets. A large compact core is composed of highly conserved hydrophobic amino acids, consistent with their expected structural role. The carboxyl and amino termini are at the opposite ends of the unit, which is 3.8 nm long. It has been proposed that the structure of other SCR is very similar, with the conserved

residues providing the structural framework for variable amino acids. These variable residues could be involved in specific interactions in much the same way as the different immunoglobulin (Ig) domains can fulfil a varied number of binding and functional roles (Reid and Day, 1989).

CHAPTER 3

COMPLEMENT AND IMMUNE RESPONSES

3.1. Early studies

Since the early 1970s it has become increasingly apparent that proteins of the complement system can influence different stages of the immune response. But, the mechanisms by which complement regulates immune responses remain to be clarified.

The first experimental evidence for the role of complement in thymus-dependent antibody production in vivo came from experiments where mouse C3 was depleted with cobra venom factor (CVF) (Pepys, 1972, 1974). CVF does not affect the total number of cells in the spleen or lymph nodes, the proportions of cells with different surface markers including Ig, Fc receptors, and C3 receptors, or the functional capacity of the different cell populations. T cells in CVF-treated animals respond normally to thymus-dependent antigens. But, antibody response in these mice was depressed (Pepys et al., 1976). It was subsequently found that CVF treatment also completely inhibits follicular localization of antigen-antibody complexes. However, once immune complexes have become localized on the surface of dendritic cells in the germinal

centres (GC), in vivo depletion of complement by CVF does not dislodge them (Papamichail et al., 1975). In contrast, the induction of tolerance to thymus-dependent antigens and thymus-independent antigens is unaffected by CVF (Pepys and Taussig et al., 1974). Thus, if complement does play a role in the induction of antibody production, it is likely that the complement component C3 and the receptors for C3 on lymphocytic cells are involved.

These speculations have been strengthened by studies of genetic deficiency models. Individuals genetically deficient in specific complement components allow the role of complement in the induction of immune responses to be addressed directly in vivo, without the disadvantages of de complementation. They also allow the classical and alternative pathways of complement activation to be differentiated. It was found that the absence of C3 or the components of the classical pathway C3 convertase seriously impaired immune responses. This has been clearly shown in cases of genetic deficiencies in C2 (Böttger et al., 1985), C3 (O'Neil et al., 1988) and C4 (Jackson et al., 1979; Ochs et al., 1983) described in humans, guinea-pigs, and dogs. In contrast, humans or animals with deficiencies of C5, C6 and C7 usually have normal immune responses (Martinelli et al., 1978; Ochs et al., 1986).

3.2. Current knowledge

In recent years, although possible modulatory functions of C1q, Ba, Bb, and C5a in immune responses have also been suggested, the bulk of experiments have dealt with the immunoregulatory role of C3 and its cleavage products (Erdei et al., 1991). It now appears that C3 is necessary both to mount normal antibody responses and for the induction of immunological memory (Böttger and Bitter-Suermann, 1987). The effects of C2 and C4 deficiency in terms of immunoregulation may be a reflection of the lack of C3 activation. There are good reasons to believe that C3 is more important than other complement proteins. First, C3 is the most abundant complement protein in serum and other body fluids. Second, C3 plays a pivotal role in both the classical and alternative pathways. Third, when activated, C3 will be split into a variety of smaller fragments such as C3b, iC3b, C3dg. These fragments can bind to Ag-Ab complexes and/or interact with their corresponding receptors present on the surfaces of different cells. The effects of these interactions have a diverse spectrum of consequences, depending upon the types of receptors and cells involved. It has been found that there are at least four types of receptors for C3-derived fragments (shown in Table 1.1). CR3 and CR4 are mainly involved in cell-

cell adhesion, or the clearance of immune complexes by phagocytic cells (Sim et al., 1987; Becherer et al., 1989). Studies on the immunoregulatory capacity of CR1 and CR2 have attracted much attention in the past decade.

Although CR1 is found on cells involved in the immune response, i.e. on all B cells, some T cells and most antigen-presenting cells, its regulatory effects on the immune response are controversial. Possibly these effects are exerted in an indirect way. Stimulation of human monocytes with C3b induces the production and release of IL-1 (Bacile et al., 1990). IL-1 is mitogenic for T cells that express specific receptors for this lymphokine and it stimulates B cell growth. Direct cross-linking of CR1 on human B cells does not induce a proliferative or differentiation response, nor does it cause the release of intracellular calcium. However, a signal may be presumed to occur since cross-linking of CR1 does lead to co-capping between CR1 and surface immunoglobulin (Tsokos et al., 1988). Perhaps more relevant to its effects on immune responses in vivo are the findings of enhancement of antibody production in vitro by pre-activated B cells in the presence of polyclonal anti-CR1 antibodies. This effect was not observed consistently when a monoclonal anti-CR1 antibody was

used (Tedder et al., 1986). The function of CR1 on T cells is largely unknown.

Data are accumulating about the regulation of immune responses by complement receptor type 2 (CR2). For instance, CR2 serves as a receptor on human B cells for mitogenic EBV which can transform B cells from the resting stage to a lymphoblastoid phase (Tosato, 1987). Calcium mobilization could be induced by EBV binding, and this effect was also accompanied by protein kinase C translocation as well as by an increase in production of phosphatidylinositol metabolites (Dugas et al., 1988). Lipopolysaccharide-activated murine B cells proliferate and mature into immunoglobulin-secreting cells after exposure to insoluble multimeric forms of C3d (Melchers et al., 1985). Certain monoclonal and polyclonal antibodies to CR2 also induce B cell proliferation and Ig secretion in the presence of T cells or T cell factors (Mittler et al., 1983; Nemerow et al., 1985). C3dg fragments or multivalent synthetic peptides corresponding to the CR2-binding site on C3d support growth of human CR2-positive lymphoblastoid B cell lines (Hatzfeld et al., 1988; Servis and Lambris, 1989; Tsokos et al., 1990; Lyamani et al., 1991). Furthermore, synergistic interaction between CR2 and sIgM on B cells increases the free intracellular calcium concentration

(Carter et al., 1988). This observation indicates that although the cytoplasmic domain of CR2 itself is too short, ligating of CR2 on B cells can deliver stimulatory signals through sIgM. Taken together, these data suggest that C3d and CR2 are likely the key complement components of importance in the immune response.

CHAPTER 4

AIMS AND OBJECTIVES

With the advent of modern techniques such as DNA recombination and monoclonal antibody methodology, much progress has been made in the area of complement research. Current interest is focused in three major areas: 1) regulation of complement activation, 2) mechanisms of host cell protection, and 3) roles of complement and complement receptors in the immune response.

This project initially began with studies on genetic polymorphisms of the regulators of complement activation. Later, the objective was expanded to evaluate the immunological functions of complement receptors, especially the CR2 molecule, in the immune response.

Therefore, the particular aims of the studies reported in this thesis have been:

- 1) To simplify the procedure for typing complement factor H, and technically improve the reliability of the procedure.

- 2) To examine the distribution of variants of factor H in people with autoimmune diseases such as rheumatoid arthritis.
- 3) To estimate factor I allele frequencies in a Caucasian population, and to compare the data with those obtained in Asiatic populations.
- 4) To identify polymorphism of complement receptor type 2 (CR2) at the protein level.
- 5) To characterize the binding epitopes of CR2 variants by flow cytometry.
- 6) To establish an animal model for evaluation of CR2 function in primary and secondary antibody responses.
- 7) To compare immunosuppression induced by cobra venom factor (CVF) with that induced by anti-CR2 monoclonal antibodies.

CHAPTER 5

MATERIALS AND METHODS

5.1. Factor H typing

5.1.1. Blood samples

Blood was collected from family members or single individuals in the local community, by venipuncture into Vacutainer tubes (Becton Dickinson, Rutherford, NJ., USA). Sera were separated, labelled, and stored at -70°C. Samples from patients with rheumatoid arthritis were collected by Dr. Martin at the Rheumatic Disease Unit, St. John's. All patients met the American Rheumatology Association (ARA) criteria for classical or definite rheumatoid arthritis (Ropes et al., 1958).

Whenever a sample was used, it was thawed quickly in a 37°C waterbath, kept on ice while being used, and thereafter refrozen as soon as possible.

5.1.2. Detection of factor H phenotypes

Isoelectric focusing was carried out according to the methods of Rodriguez de Cordoba and Rubinstein (1984), with the modification that serum factor H was not immunoprecipitated prior to focusing. A vertical slab polyacrylamide gel (160 X 1600 X 1 mm) containing 1.7% NP-40 (Sigma, St. Louis, MO., USA), 8 M urea (Bio-Rad (Canada), Mississauga, Ont., Canada), 2% ampholine (LKB, Piscataway, NJ., USA) mixture (pH 3.5-10 : 4-6 : 5-8 = 1 : 4 : 4) was used. 5 ul of serum pre-treated with neuraminidase were applied to each slot (10 ul of serum was incubated at room temperature overnight with 1 U of neuraminidase type V, Sigma). 0.01 M NaOH (Fisher Scientific Ltd., Montreal, Que., Canada) and 0.01 M H_3PO_4 (BDH Canada Ltd., Toronto, Ont., Canada) were used as cathode and anode solutions, respectively, and isoelectric focusing was conducted on a PROTEIN II apparatus (Bio-Rad) with 400 V overnight. The proteins were then electroblotted onto a nitrocellulose membrane according to the instructions of the manufacturer (Bio-Rad). The factor H protein was detected using goat-anti-human factor H ($\beta 1H$) (at 1:300 dilution; Atlantic antibodies, ME., USA) followed by peroxidase-conjugated rabbit anti-goat IgG (at 1:600 dilution; Atlantic antibodies). The protein

bands were developed with 3,3'-diaminobenzidine (Sigma) and H_2O_2 (Sigma) as substrates.

5.2. Factor I typing

5.2.1. Blood samples

Blood was collected from people from the local community. A small number of samples were collected specifically from Chinese individuals living in the St. John's area. Sera were separated, labelled, and stored at $-70^{\circ}C$. Whenever a sample was used, it was thawed quickly in a $37^{\circ}C$ waterbath, kept in ice while being used, and thereafter refrozen as soon as possible.

5.2.2. Detection of factor I variants

A horizontal, ultra thin-layer polyacrylamide slab gel, previously described by Nakamura and Abe (1985), was used for factor I typing, with a slight modification, i.e. a mixture of ampholine (LKB) pH 3.5-10 and pH 5-8 in a ratio of 1:2 was used instead of pH 3.5-9.5 alone. Isoelectric focusing was performed with a LKB 2103 Power Supply on a Pharmacia Flatbed Apparatus electrophoresis unit (Pharmacia, Uppsala, Sweden).

For each slab gel, a paper strip was placed at each end. One strip was wetted with 1.0 M H_3PO_4 (anode) and another with 1.0 M NaOH (cathode). At first, the gel was prefocused at 400 V for 30 min without samples. Then the samples, which had been treated with neuraminidase as described for factor H typing, were applied to the gel surface with Whatman 3 MM filter paper (Fisher) at a distance of 1.0 cm from the anode end. The gel was focused at 800 V for 1 h, then the sample application pieces were removed. The focusing was continued at 1,000 V for 3 h. The proteins were electroblotted onto a nitrocellulose membrane. To detect factor I patterns, two identically reacting anti-human factor I antisera, raised in goat or sheep respectively (Wako Pure Chemical Industries, Osaka, Japan; ICN Biochemicals, Costa Mesa, CA., USA), were employed. The bands were visualized with a second enzyme-labelled antibody and enzyme substrates, 3,3'-diaminobenzidine and H_2O_2 .

5.3. CR2 typing

CR2 is a membrane receptor expressed on B lymphoblastoid cells, as well as on peripheral mature B lymphocytes. Since B cell lines usually express more CR2 than peripheral B cells, B cell lines were the first choice for CR2 typing. Healthy, growing B cell lines were radioactively labelled with ^{125}I , and

than the immunoprecipitates of cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). A flow diagram of these procedures is shown in Fig. 5.1.

5.3.1. Cell lines

B lymphoblastoid cell lines were established from peripheral B lymphocytes transformed with Epstein-Barr virus. In brief, peripheral mononuclear cells were prepared from heparinized blood by density centrifugation over Ficoll-Hypaque (Lymphoprep, Cedarlane, Hornby, Ont., Canada). T lymphocytes were removed by cytotoxic treatment with Lympho-Kwik (One Lambda, Los Angeles, CA., USA). Afterwards, the enriched B lymphocytes were first infected with an appropriately diluted culture supernatant of B95-8 (a transforming strain of EBV), then dispensed (200 μ l per well) to a 96-well flat-bottom tissue culture plate (Nunc, CDN Life Technologies, Burlington, Ont., Canada). After 1-2 weeks of culture at 37°C in a CO₂ incubator, immortalized B cells were transferred to a 24-well plate (Nunc), and finally expanded in a small flask (50 ml) (FALCON, Becton Dickinson). Later, B cell lines that were growing well were frozen and stored in liquid nitrogen. The cells could be recovered by thawing

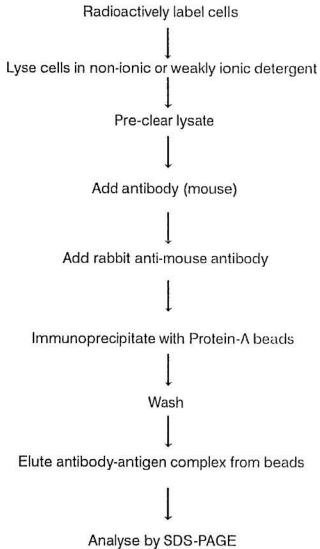


Figure 5.1. Flow diagram of CR2 immunoprecipitation.

quickly at 37°C and re-culturing in fresh medium [RPMI 1640, ICN (see below)].

As controls, the CR2-positive B lymphoblastoid cell line Raji (a Burkitt's lymphoma cell line) and the T lymphoblastoid cell line Jurkat were used. Both of these lines were obtained from American Type Culture Collection (ATCC), MD, USA.

5.3.2. Cell labelling and immunoprecipitation

Healthy cells were allowed to grow to a desired density in culture medium RPMI 1640 (ICN) containing 100 IU/ml penicillin (ICN), 100 ug/ml streptomycin (ICN), 2 mM L-glutamine (ICN) and 10% v/v heat inactivated fetal calf serum (FCS) (ICN). The cells were harvested by centrifugation, and washed with ice-cold PBS (Sodium Phosphate Buffer, pH 7.2-7.4) three times. The labelling of cell membranes with Na¹²⁵I (ICN or Amersham Canada Ltd., Oakville, Ont., Canada) was performed in a fumehood by the IODO-Gen method recommended by the manufacturer (Pierce, Rockford, IL., USA). The labelled cells were washed with ice-cold PBS, and lysed with an appropriate volume of lysis buffer [(10 mM Tris-HCl (Sigma), pH 7.4, 1 mg/ml BSA (Sigma), 1% w/v NP-40 (Nonidet P-40) (Sigma), 1 mM PMSF (phenylmethanesulphonyl fluoride) (Sigma), 150 mM NaCl

(Fisher), 1 mM EDTA (diethylenediamine tetraacetic acid)) (Sigma). The lysate was centrifuged at 10,000 x g for 2 min. and the supernatant was used for immediate immunoprecipitation, or was stored at -70°C for experiments the next day. All manipulations were performed on ice.

Before immunoprecipitation of CR2, the lysate of each cell line was pre-cleared by incubation with normal rabbit serum (5 ul for 200 ul lysate) and subsequent incubation with Protein A-Beads (Protein-A Agarose or Protein A-Sepharose, Sigma or Pharmacia), followed by centrifugation. The supernatant was then immunoprecipitated by adding 10 ug of a specific anti-human CR2 monoclonal antibody (HB-5). HB-5 monoclonal antibody has been purified by affinity chromatography on a Protein-A Agarose column (Sigma) from the culture supernatant of its clone purchased from ATCC. No additional protein bands were seen on SDS-PAGE, after staining with Coomassie blue (Sigma). The reaction of HB-5 with the lysate was carried out in ice for at least 3 h. To ensure maximal precipitation, a polyclonal rabbit anti-mouse IgG (BIO/CAN, Mississauga, Ont., Canada) was added afterwards, and the incubation continued for 2 h. Finally, 50 ul of washed Protein A-beads (Pharmacia or Sigma) were added and the precipitates, bound to the beads, were harvested by centrifugation. The precipitates were then

washed extensively: 1) with wash buffer (10 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF) plus 0.5 M NaCl; 2) with wash buffer plus 0.1% SDS; 3) with 10 mM Tris-HCl pH 7.4, 0.1% NP-40. Thus, non-specific, bound proteins would be washed away. The CR2 precipitates were stripped from the beads by adding 50 μ l sample buffer [0.08 M Tris, 10% glycerol (Sigma), 2% SDS (Bio-Rad), 0.01% bromophenol blue (Sigma) and 0.1 M β -mercaptoethanol (Sigma)] and boiling for five minutes. The supernatant was then ready to load onto an SDS-polyacrylamide gel.

5.3.3. CR2 purification

CR2 purification from labelled lysate was performed on an affinity chromatography column using HB-5 coupled to agarose beads (Affi-Gel Hz immunoaffinity kit, Bio-Rad). The radioactivity and protein concentration of each fraction (100 μ l) were measured by a γ counter (Gamma 310 system, Beckman, Fullerton, CA., USA) or by a spectrophotometer at 280 nm (DU Series 60, Beckman), and the peak fraction was then subjected to SDS-PAGE analysis and autoradiography.

5.3.4. SDS-PAGE analysis

One dimensional 7.5% SDS-PAGE under reducing conditions was employed for analysis of CR2 variants. The gel was cast according to the conventional method of Laemmli (1970). In brief, the 7.5% separation gel was set first, and gently overlaid with water. After polymerization, the 3% stacking gel was poured on the top of the separation gel, and the "comb" was placed into the stacking gel, making sure no bubbles were trapped in the sample wells. When the gel became hard, the "comb" was removed and the sample wells were washed with water. The samples were then applied to the wells (20-50 μ l per well). The running buffer (0.025 M Tris, 0.192 M glycine (Sigma), 0.1% SDS, pH 8.3) was loaded into the top and bottom of the electrophoresis apparatus (Protean II or Mini Protean II, Bio-Rad). Electrophoresis was conducted until the dye marker nearly reached the gel bottom. Pre-stained molecular weight markers (Bio-Rad) were run on the same gel. After separation, the gel was removed from the apparatus, and dried. The radiolabelled CR2 precipitates were located by autoradiography.

5.4. Characterization of epitopes on CR2 variants by flow cytometry

To define possible changes in specificity associated with CR2 structural variants, the ligand binding of CR2 among the different B cell lines was analyzed by flow cytometry. These experiments involved the isolation and biotinylation of EBV particles, and an EBV binding assay under the conditions of either non-inhibition or inhibition with monoclonal antibodies.

5.4.1. Isolation and labelling of EBV

The isolation and labelling of EBV particles were achieved by following step by step the method described by Nemerow & Cooper (1981) and Inghirami et al. (1988). In brief, marmoset lymphoma B95-8 culture supernatant containing the virus was collected and cleared of debris by centrifugation at 1,500 x g for 45 min. Virus was separated by two sequential cycles of pelleting at 100,000 x g at 4°C for 60 min and by subsequent banding in a 5 to 30% dextran T-10 (Pharmacia) discontinuous gradient prepared in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4. The single interface layer containing the virus particles was collected. The particles were pelleted and suspended in

carbonate buffer [0.05 M carbonate (Fisher), 0.135 M NaCl, pH 8.8]. The virus was incubated with dimethyl sulfoxide containing N-hydroxysuccinimido-biotin (Sigma). Free biotin was removed by dialysis against PBS (pH 7.4) at 4°C. Aliquots of biotinylated EBV (50 ul/tube) were stored at -70°C until use.

5.4.2. Flow cytometry

Flow cytometry analysis was performed on a FACStar Plus (Becton Dickinson, Palo Alto, CA., USA). Anti-CR2 monoclonal antibodies, OKB-7 (Ortho, Raritan, NJ., USA), HB-5 (ATCC) and B2 (Becton Dickinson) or biotin-EBV were used. 10^5 cells were incubated with 5 ul of biotinylated EBV or 5-10 ug of anti-CR2 mAb for 1 h at 4°C in culture medium RPMI 1640 with 2% FCS. The cells were then washed three times in the same medium and incubated for 1 h at 4°C with fluorescein-isothiocyanated streptavidin (FITC-Avidin) (Sigma) or with FITC-conjugated goat anti-mouse IgG and IgM (Becton Dickinson), respectively. For inhibition tests, there were two kinds of experimental approaches. First, the cells were preincubated with 10 ug of anti-CR2, either OKB-7 or HB-5, and washed before the labelled EBV was added. Second, prior to addition to the cells, the labelled EBV was incubated with an excess of monoclonal

antibody 72A1. 72A1 is specific for gp350/220 of EBV. (The clone 72A1 was obtained from ATCC, the mAb (IgG₁) was purified on an affinity column). The inhibition of EBV binding was measured after reaction with FITC-avidin (Sigma).

5.5. Characterization of the role of CR2 in immunoregulation

Complement receptors, especially CR2, seem to be key components of complement involved in immunoregulation. To further define such a function of CR2 in vivo, an animal system was established in mice.

5.5.1. Mice

Female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA., USA) and were used at 8-16 wk of age. Animals were maintained in accordance with institutional recommendations.

5.5.2. Antigen

Purified, divalent F(ab')₂ fragments of goat IgG, purchased from Cappel (West Chester, PA., USA), were used as Ag throughout all the experiments. The F(ab')₂ fragments were

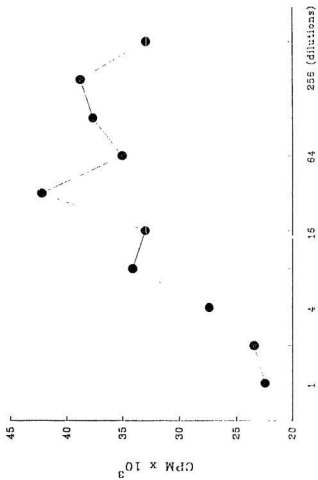
from a goat antiserum specific for the Fc regions of all rat IgG isotypes. In order to eliminate cross-reaction with murine Ig, the Ag [F(ab')₂] was passed through a mouse serum-agarose column (Sigma) before use.

5.5.3. Antibodies

Three different rat mAbs, 7G6 (IgG_{2b}), 7E9 (IgG_{2a}) and 8C12 (IgG_{2c}) with specificity for murine CR were used (Kinoshita et al., 1988). As previously described (Kinoshita et al., 1990; Molina et al., 1991), 7G6 can completely inhibit the C3d-mediated rosette formation whereas 7E9 can not, and therefore 7G6 is functionally defined as an anti-CR2 antibody although both 7G6 and 7E9 also bind to CR1. 8C12 is monospecific for murine CR1. As a control mAb, RA3-6B2, a rat IgG_{2b} against CD45 (B220) was used. Like CR2, the B220 molecule is a lineage marker for pre-mature and mature B cells (Thomas, 1989). Purified 7G6, 7E9 and 8C12 were gifts of Dr. Kinoshita (Osaka University Medical School, Japan) while RA3-6B2 was purified on a Protein-A Agarose column (Sigma) from culture supernatant of its clone, kindly provided by Dr. M. Dailey of The University of Iowa. On SDS-PAGE with reducing condition, purified RA3-6B2 mAb gave only two bands, corresponding to IgG heavy and light chains.

5.5.4. Preparation of an immune complex form of Ag

As mentioned above, Ag was divalent goat F(ab')₂ against rat IgG while monoclonal antibodies used were rat IgG. Therefore, free Ag would be converted to a complex form of Ag if mixed with either mAb. To obtain maximal amounts of immune complexes for injection a precipitation curve was established by mixing varying amounts of goat F(ab')₂ with constant amounts of rat IgG. The rat IgG had been ¹²⁵I-labelled by the IODO-GEN method (Pierce), and separated from non-bound NaI on a Sephadex G-25 (Pharmacia) column. It had a specific activity of 1.2×10^5 cpm/ug. After incubation overnight at 4°C, formed immunocomplexes were washed with cold-PBS three times, and then transferred into fresh tubes for measurement of radioactivity in a γ counter (Beckman) (Fig. 5.2). The equivalent ratio between Ag and Ab was determined by assuming that the amount of precipitate is in proportion to the total immune complexes formed (soluble and insoluble). The immune complex of Ab-Ag prepared this way was used in all further experiments. When Ag was required in various doses, a single, large volume of immunocomplexed Ag was prepared, and then diluted for immediate use.



5.5.5. Protocols for immunization

There were two protocols for immunization.

1) The protocol used for this group of experiments will be described in detail in RESULTS. Briefly, mice were first treated either with a single injection of 200 ug mAb or pretreated with four injections of 5 ug CVF over a period of 24 h (for a total of 20 ug CVF). The primary antibody response in all mice was elicited by tail vein administration of 200 ug antigen in IFA (incomplete Freund's adjuvant, Sigma) in 0.2 ml PBS. Specific IgM and IgG responses were assayed in individual mice by ELISA at different times. Forty days later, secondary responses were boosted with an optimal dose of antigen, 6.25 ug of free Ag in PBS. This had previously been determined as a minimal requirement for induction of a secondary response. Specific IgG production was checked five days later.

2) This protocol had three phases. First, the primary antibody response was elicited in all the mice using the same dose of antigen, i.e. 200 ug Ag plus IFA in 0.2 ml PBS were administrated intravenously in the tail. Second, the primed mice in randomly divided groups were boosted with decreasing doses of antigen alone so as to find the minimal requirement

of antigen for induction of a secondary response. Third, the antigen was given in different forms (free vs complexed) and the ability of each form to induce a secondary response was compared. Various amounts of Ag were given to boost the secondary responses.

5.6. Blood sampling of mice

Blood samples were collected individually by puncturing the tail vein with a #27½ needle (Becton Dickinson). Drop by drop, up to 100 ul of blood could be obtained from a single mouse at a time. The frequency of sampling varied between experiments (see RESULTS). Serum was separated and stored at -70°C.

5.7. ELISA

Direct sandwich ELISA was employed for detection of antibody responses. The method, with slight modifications, was adopted from a standard procedure, recommended by Kemeny (1991). Briefly, 96-well plates (U type; ICN) were coated with Ag solution (10 ug/ml, 50 ul/per well) in 0.1 M bicarbonate buffer pH 9.6 overnight and then blocked with 0.05% Tween 20 (Sigma) in PBS, followed by three washes with PBS. All sera were diluted with PBS containing 1% BSA. To each well 100 ul

of diluted serum was added and incubated for 1 h at room temperature. The plates were washed with PBS three times. Alkaline phosphatase (AP)-labelled anti-mouse IgM (μ chain specific) (Cappel) or IgG (Fc_γ specific) (Cappel) was added, and the plates were incubated for 1 h at room temperature, followed by three washes. AP activity was measured by the modified method of Sorimachi and Yasumura (1986), using 4-aminoantipyrine (Sigma) as substrate. Optical density was read on a Bio-Rad microplate reader 3550 at 490 nm. For quantification of specific IgG, ELISA was performed as above, with the addition of a set of standard wells. Instead of Ag, a goat anti-mouse Ig antiserum (Sigma, diluted 1/400) was used to coat the standard wells. The standards were purified mouse IgG (Sigma), diluted to 250, 125, 25, 2.5 and 0.25 ng per well, or purified mouse IgM (Sigma), diluted to 25, 12.5, 2.5, 0.25 and 0.025 ng per well. The standard curves (semi-log) were automatically plotted and the IgG or IgM amounts of samples calculated by the microplate reader's software (Bio-Rad). All samples and standards were in duplicate. For IgG subtypes, Mouse Typer Sub-Isotyping kit (Bio-Rad) was used as described by the manufacturer.

6. Statistics

Statistical analysis was performed with InStat 1.0 software (GraphPAD, San Diego, CA., USA).

6.1. Chi-square

The association of phenotypic variants with diseases were determined by Fisher's exact test.

6.2. Analysis of variance (ANOVA)

One-way ANOVA was used in experiments where there were more than two groups of data. It should be stressed here that for the ELISA data obtained from the secondary antibody responses, the mean was the antilog of the mean of the logarithm of the original variable, since the arithmetic means in this case do not fit the normal distribution.

6.3. Student's t test

Student's t test was applied to determine if there was a difference between the means of two groups of samples.

CHAPTER 6

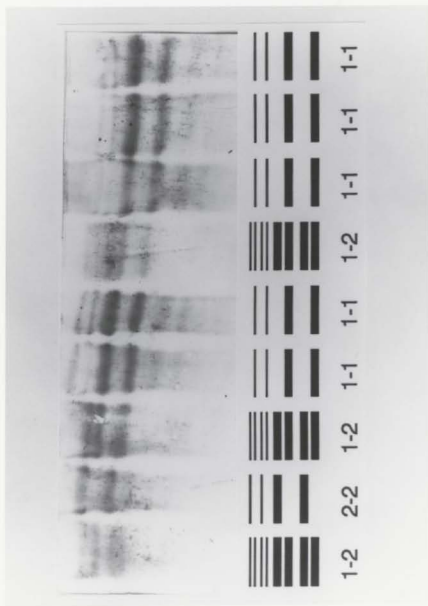
RESULTS

6.1. Factor H typing

Phenotypes: Samples from 129 local unrelated healthy individuals were typed by isoelectric focusing of neuraminidase-treated serum. A representative set of patterns is shown in Fig. 6.1. Pattern 1-1 and pattern 2-2 each contained two major bands in the pH interval 6.5 to 7.0. This was demonstrated by measuring the pH of 1 cm-wide gel slices each eluted with 1 ml distilled water overnight (Fig. 6.2). The bands of pattern 1-1 are focused at a slightly more basic position than those of pattern 2-2. In pattern 1-2 these four bands appear as doublets, as would result if pattern 1-1 was superimposed on pattern 2-2. According to these observations, two forms of factor H, FH 1 and FH 2, would be responsible for the three patterns, both forms coexisting in pattern 1-2. The patterns are reproducible.

Inheritance and allele frequencies: Co-dominant segregation of these three forms of factor H was demonstrated in 13 families with 58 children (Table 6.1). No exception was found

Figure 6.1. Immunoblotting patterns of factor H phenotypes



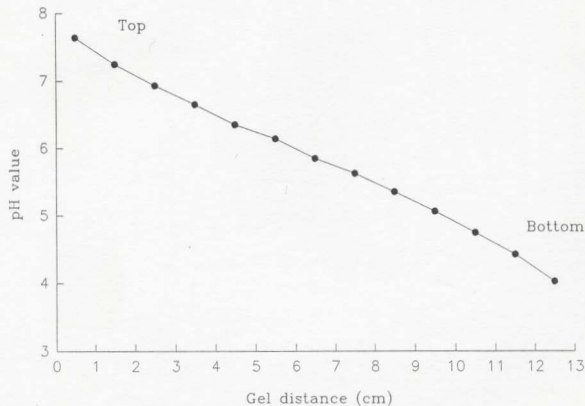


Figure 6.2. The pH gradient of isoelectric focusing gel for factor H typing. At the end of a run the gel was sliced, and the pH value for each slice was measured.

Table 6.1. Factor II phenotypes in 13 families with 58 children

Parental phenotypes	Number of families	Children's phenotypes		
		FH 1-1	FH 1-2	FH 2-2
1-1 x 1-1	0	-	-	-
1-1 x 1-2	6	18	9	-
1-1 x 2-2	3	-	13	-
2-2 x 2-2	1	-	-	1
2-2 x 1-2	1	-	2	2
1-2 x 1-2	2	3	6	4
Total	13	21	30	7

to the rule for autosomal co-dominant inheritance. No evidence of null genes was found by studying these 13 families, and no more than two different forms of factor H were observed in the same individuals. These results indicate that there are two common co-dominant alleles at the factor H gene locus, FH 1-1 and FH 2-2. Pattern 1-1 is the homozygous $FH*1/FH*1$; pattern 2-2 is the homozygous $FH*2/FH*2$; and pattern 1-2 is the heterozygous $FH*1/FH*2$. The allelic frequencies of factor H in Newfoundland were calculated from direct gene counting under the assumption of absence of a null gene. These frequencies were as follows: $FH*1 = 0.597$ and $FH*2 = 0.403$. The expected frequencies of the different phenotypes calculated from these allele frequencies did not differ from the observed values, indicating the population studied to be in Hardy-Weinberg equilibrium (Table 6.2).

Reliability of this method for factor H typing: Some Newfoundland samples were also typed blind by Dr. Rodriguez de Cordoba at New York by his method. The results were conclusive, confirming that the two methods of typing produced comparable data.

Table 6.2. Distribution of phenotypes and gene frequencies of human factor II.

Phenotypes	Observed No.	Percentage	Expected No.	χ^2
II 1-1	50	38.7	46	0.347
II 1-2	54	41.9	62	1.032
II 2-2	25	19.4	21	0.762
Total	129	100.0	129	2.141

$F_{II}^* 1 = 0.597$ d.f. = 1
 $F_{II}^* 2 = 0.403$ $0.10 < P < 0.25$

Factor H typing in patients with rheumatoid arthritis: In addition to a general population sample, a group of 111 patients with an autoimmune disease, rheumatoid arthritis, was also typed for factor H. The distributions of factor H phenotypes in the healthy group and the group of patients are compared in Table 6.3. The allele frequencies in patients were: $FH*1 = 0.568$ and $FH*2 = 0.432$. No association of a factor H phenotype or variant with the disease was found by Chi-square analysis. However, there was a significant decrease in heterozygote frequency in the group of patients ($\chi^2 = 5.8$, d.f. = 1, $0.01 < p < 0.025$). The reason for this is not clear.

6.2. Factor I typing

Factor I phenotypes: Polymorphism of factor I was first demonstrated in Japanese (Nakamura et al., 1985). It was observed that there were three different common patterns (two homozygous patterns and one heterozygous pattern). Homozygous individuals have two major bands and some acidic minor bands, and heterozygous individuals have the composite of two homozygote types. The three common phenotypes were designated IF B (for more basic pI), IF A (for more acidic pI), and IF AB, respectively. Family studies supported the hypothesis that factor I polymorphism was controlled by autosomal co-dominant

Table 6.3. Comparison of factor II phenotypes in healthy controls and patients with rheumatoid arthritis

Phenotypes	<u>Controls</u>		<u>Patients^a</u>	
	No.	%	No.	%
FI 1-1	50	38.7	42	37.8
FI 1-2	54	41.9	42	37.8
FI 2-2	25	19.4	27	24.3
Total	129	100.0	111	100.0

$$\chi^2 = 0.93, \text{ d.f.} = 2, P = 0.63$$

$$^a \chi^2 = 5.82, \text{ d.f.} = 1, 0.01 < P < 0.025$$

Mendelian inheritance at a single locus with two common alleles. These findings were confirmed by later studies, conducted in the western part of Japan (Nishimukai et al., 1986), and among other populations in Eurasia (Yuasa et al., 1988). Isoelectric focusing and immunoblotting were employed in all these studies, with slight modifications.

In an initial experiment, six samples were selected for treatment with neuraminidase for different durations to see the effect of the enzyme on the allotype pattern. As shown in Fig. 6.3, the one to two-hour treatment and the overnight treatment at room temperature yielded distinct allotype patterns for the same samples. Clearly, the positions of bands (major and minor) after overnight treatment were further shifted towards the cathode. Although treatment for less than 2 h produced clear bands, for more stable patterns overnight treatment was preferred.

A new variant of factor I: Using this technique, a study was carried out in a group of 288 unrelated Caucasian individuals living in the St. John's area. A representative electrofocusing pattern is shown in Fig. 6.4. Surprisingly, no single individual heterozygous for factor I was found. Later, one person who is a heterozygous carrier of a new factor I

Figure 6.3. Factor I patterns after neuraminidase treatment for different time periods. From left to right, 1-6: overnight; 7-12: two hours; 13-18: one hour.



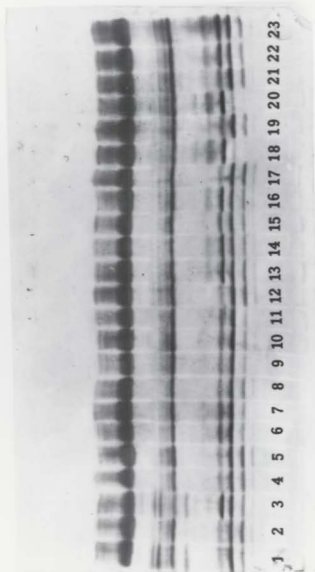
Figure 6.4. A representative picture of the identical factor I pattern from 288 Caucasian individuals.

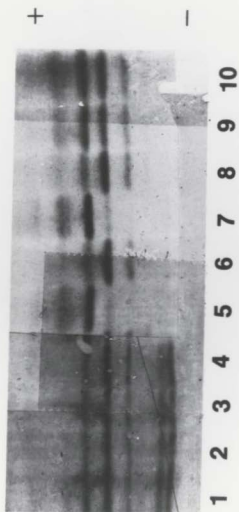


variant was found. There were some doubts about the method's reliability, and all doubts were not dismissed until a panel of factor I reference samples, kindly provided by Drs. Nakamura (Tokyo Women's Medical College, Tokyo, Japan) and Yuasa (Tottori University School of Medicine, Yonago, Japan), were included in a parallel study. In addition, 17 serum samples from Chinese students at Memorial University were collected for factor I typing. Among these Chinese samples were 7 individuals who were IF AB heterozygous, as shown in Fig. 6.5.

The new variant of factor I referred to above was found in a Caucasian. It was confirmed in samples from the mother of the proband and two siblings. All presented the same factor I pattern as that of the proband (Fig. 6.6). A sample from the father was not available. The finding of a new variant in this family is further supporting evidence for autosomal codominant inheritance. The new variant band was focused in a much more basic region than the common IF B. It was designated IF B1 at the Vth Complement Genetics Workshop and Conference, Mainz, Germany, 1989. Polymorphic factor I variants all have the same molecular weight as judged by SDS-PAGE (data not shown).

Figure 6.5. Factor I phenotypes of 17 Chinese sera (lanes 1-17) and 6 reference samples (18-23). Homozygous IF A type: lanes 18 and 20; homozygous IF B type: lanes 1-2, 4-5, 8-12, 17, 19 and 21; heterozygous IF AB type: lanes 3, 6-7, 13-16, 22-23.





The predominant factor II gene in the population studied is *IF*B* with a frequency of over 0.999. The frequency of the variant *IF*B1* is 0.001, and *IF*A* is lacking.

6.3. CR2 typing

Preliminary studies: EBV-transformed human B cells were surface-labelled with ^{125}I and the membrane CR2 proteins that were immunoprecipitated by monoclonal anti-CR2 (HB-5), were analyzed by SDS-PAGE and autoradiography. Two reference cell lines, the CR2-positive B lymphoma cell line Raji, and the CR2-negative T lymphoblastoid cell line Jurkat, were used in a preliminary experiment. As shown in Fig. 6.7, no radiolabelled material was observed in the precipitates of Jurkat cell lysate. In contrast, the immune precipitates from Raji cell lysate yielded a heavy band of radioactivity having a molecular weight of about 140 kDa. The results were reproducible.

CR2 from labelled Raji cell lysate was purified on an affinity chromatography column (p. 83). The autoradiography band of the eluted CR2 was identical to that of the nonpurified CR2 immunoprecipitate, as presented in Fig. 6.8.

Figure 6.7. CR1 and CR2 immunoprecipitates from Raji and Jurkat cell lines. Lanes 1 (Jurkat) and 2 (Raji), CR1 precipitate by anti-CR1 mAb Yz-1; lanes 3 (Jurkat) and 4 (Raji), CR2 precipitate by anti-CR2 mAb HB-5. Jurkat is negative for both CR1 and CR2.

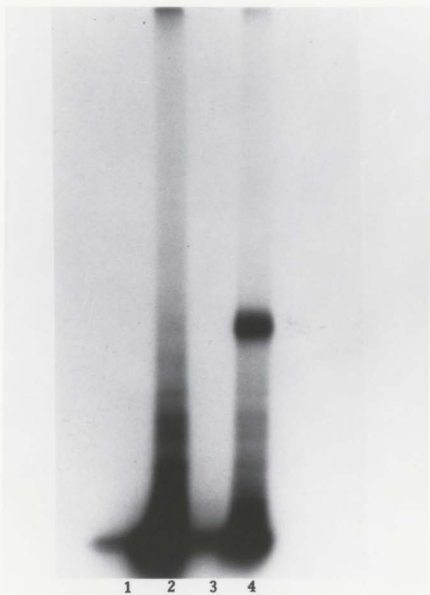
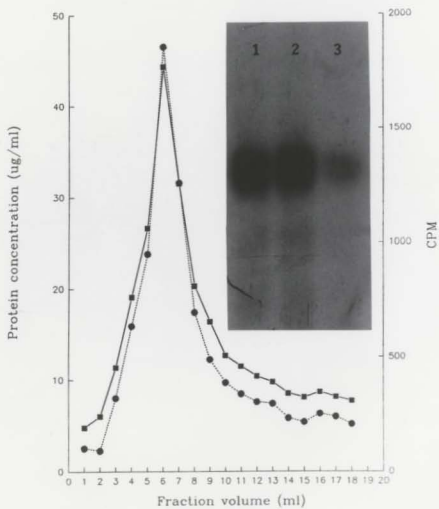


Figure 6.8. Purification of CR2 from labelled Raji lysate (● Protein concentration, ■ CPM). The inserted picture shows the SDS-PAGE bands of fractions 6 (lane 1) and 7 (lane 2), and of the unfractionated precipitates (lane 3).



Finally, the immunoprecipitated CR2 was treated with neuraminidase, and a comparison of treated CR2 with nontreated CR2 is presented in Fig. 6.9. Samples from three individuals were tested, lanes 1-3 being treated and lanes 4-6 being nontreated samples. As expected, there was only a slight decrease in molecular weight seen in all three samples after treatment.

Taken together, these preliminary data indicated that the method of SDS-PAGE analysis of radiolabelled immunoprecipitated CR2 was appropriate for CR2 phenotyping, and the reference cell lines were quite reliable.

CR2 Phenotypes: B cell lines from 63 different individuals including 9 patients with rheumatoid arthritis were analyzed by SDS-PAGE. The typical patterns are shown in Fig. 6.10. All cell lines, except cell line 5, expressed the common CR2 molecule of about 140 kDa. Cell line 5 yielded a band of 75 kDa, along with another band (indicated by an arrow in Fig. 6.10). The extra band could represent the heavy chain of surface immunoglobulins based on its molecular weight, or could be one of the components of the CR2 complex. These possibilities will be addressed later (p. 170). The cell lines 1, 2 and 7 also had the extra band of 75 kDa as well as the common CR2.

Figure 6.9. SDS-PAGE of CR2 after and before treatment with neuraminidase. Three samples were tested: lanes 1-3, treated; lanes 4-6, non-treated. Non-treated CR2 has a molecular weight of approximately 140×10^3 while neuraminidase-treated CR2 has a molecular weight of approximately 120×10^3 .

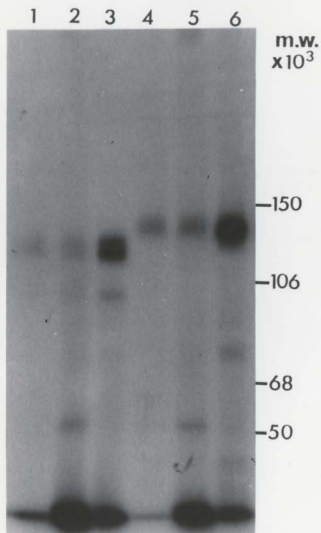
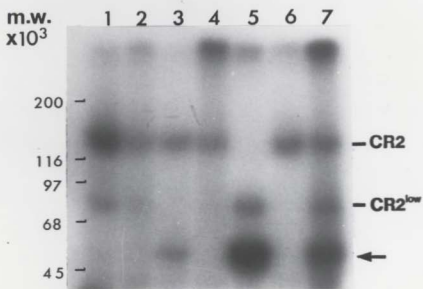


Figure 6.10. CR2 polymorphism identified by SDS-PAGE. Lane 5: CR2 L/L homozygous; lanes 1, 2 and 7: CR2 H/L heterozygous; lanes 3, 4 and 6: CR2 H/H homozygous.

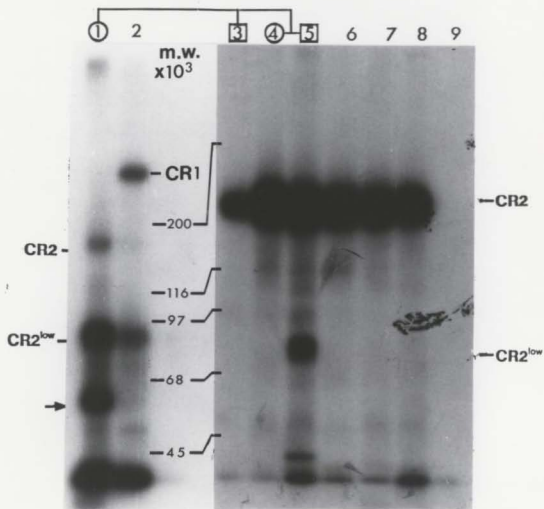


These patterns could be interpreted as follows: Cell line 5 is homozygous for the variant CR2; cell lines 1, 2 and 7 are heterozygous; and the rest of the cell lines are homozygous for the common CR2. This is the first molecular weight variant of CR2 found on the B cell surface. The variant is tentatively designated CR2 L type, the letter L standing for lower molecular weight. The common CR2 with higher molecular weight is accordingly referred to as CR2 H type.

A family study: The above interpretation was further supported by a family study (Fig. 6.11). In this family, the father (lane 5) is a heterozygous carrier of the variant CR2 (H/L) and the mother (lane 4) homozygous for the common CR2 (H/H). Two siblings were also tested, showing that the son (lane 3) is homozygous for the common CR2 (H/H), but the daughter is heterozygous (H/L). An extra band was encountered in the daughter's sample (indicated by an arrow in fig. 6.11). Because the daughter's peripheral B cells were very difficult to transform by Epstein-Barr virus under the standard conditions, the typing test was done by using her peripheral mononuclear cells (PMN).

In Fig. 6.11, are included another three cell lines (lanes 6-8) along with Jurkat (CR2-negative) (lane 9). Additionally,

Figure 6.11. Inheritance of CR2 variant L allele in a family (lane 1: Daughter; lane 3: Son; lane 4: Mother; lane 5: Father). Lane 2 is a re-precipitate of lane 1 by polyclonal anti-CR1. Lane 6-8: normal cell lines, and lane 9 is Jurkat.



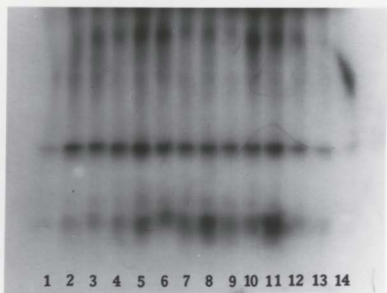
lane 2 shown here is considered a control since the same lysate was used for CR2 immunoprecipitation first (lane 1), then the resulting supernatant was re-used for CR1 precipitation (lane 2). There are two bands seen in lane 2: the top band of CR1 of over 200 kDa, precipitated with a polyclonal anti-human CR1 antibody (SEROTEC, Prince Laboratories Inc. Toronto, Ont., Canada), and the CR2 L band. The CR2 L band might be the remaining CR2 precipitates due to incomplete clearing. The possibility that polyclonal anti-CR1 cross-reacts with CR2^{low} has not been excluded.

Unfortunately, the family samples of the CR2 (L/L) homozygote described above were not available, otherwise, more information about the L variant would have been available.

There was no CR2 polymorphism due to molecular charge difference. As shown in Fig. 6.12, all the samples including an H/L heterozygous cell yielded an identical, single band on the isoelectric focusing gel.

Allele frequencies: Although the sample group tested is small (n=63), this new variant CR2 L with a molecular weight of about 75 kDa was carried by six heterozygous unrelated individuals, and one homozygous individual. The allele

Figure 6.12. Isoelectric focusing pattern of CR2 precipitates from different cell lines, including the CR2 L/H line (lane 5) and Raji (lanes 1 and 13).



frequencies for both H and L types were calculated (Table 6.4). The L frequency was over 0.01. It may be concluded that CR2 size polymorphism exists in the population studied.

6.4. Characterization of epitopes on the CR2 novel variant

Flow cytometry: Since human CR2 acts as a receptor for at least two ligands, C3d, EVB, and probably interferon- α , the newly defined CR2 variant needed to be tested for any possible changes in its specificity. Such changes could, if found, be due to loss of SCRs, or to conformational alteration. Its molecular weight (about 75 kDa) is only half of the molecular weight of the common CR2. Although the binding site for INF- α on CR2 is unclear, the region for C3d and EBV binding has been precisely localized at the first SCR. There are three mAbs with well-defined epitopes: OKB-7 recognizes the SCRs 1-2, inhibiting both EBV and C3d binding; HB-5 recognizes the SCRs 3-4; B2 recognizes the SCRs 9-14 (Fig. 2.2).

Using these three mAbs, several B cell lines with known phenotypes were first tested for expression of CR2 on the cell surface (Fig. 6.13). Jurkat is the negative and Raji the positive control. The FS 8212 is the homozygote of CR2^{low}.

Table 6.4. Distribution of phenotypes of human CR2

Phenotypes	Observed No.	Percentage	Expected No.	χ^2
CR2 H/H	56	88.9	55.3	0.009
CR2 H/L	6	0.09	7.4	0.265
CR2 L/L	1	0.02	0.3	1.633
Total	63	100.0	63.0	1.907

$CR2^* H = 0.937$ d.f. = 1
 $CR2^* L = 0.063$ $0.10 < P < 0.25$

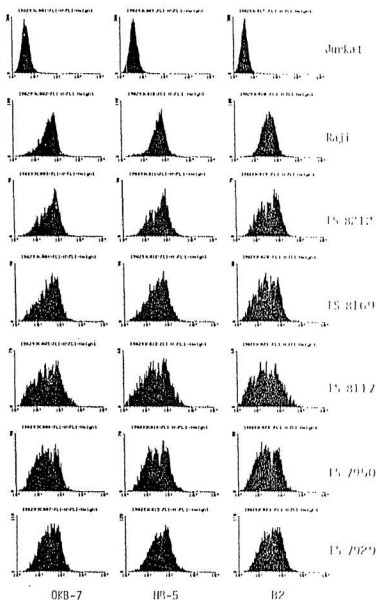


Figure 6.13. Flow cytometric profiles of cell lines stained with three different mAb to human CR2.

The three mAbs gave virtually identical flow cytometric profiles for each individual cell line but the profiles differed between the cell lines. Also, the CR2 L/L homozygous cell line, FS 8212, was positive for all the antibodies used.

In order to visualize directly the binding of biotin-EBV to CR2 on the cell surface, human B cell lines were reacted with biotin-EBV and then stained with FITC-avidin. After washing, relative amounts of EBV bound on cells were analyzed by flow cytometry. Flow cytometry depiction of a small group of cell lines randomly selected is shown in Fig. 6.14. Clearly, binding of EBV to these cell lines was extremely variable, positive cells accounting for from 25 (FS 8230) to 99% (FS 5637). The data were consistent with the profile of CR2 expression defined by using monoclonal antibodies (Fig. 6.13). Thus, an analysis of the binding property of CR2 on each cell line could only be made by its own pair comparison under inhibitory or non-inhibitory conditions.

The cell line FS 8212, which was homozygous for CR2 L/L, was chosen for the investigation of the CR2 L variant binding specificity. The experiment was carried out in several ways. Besides a direct test, as described above, three kinds of inhibitory tests were included. In the first pair of tests,

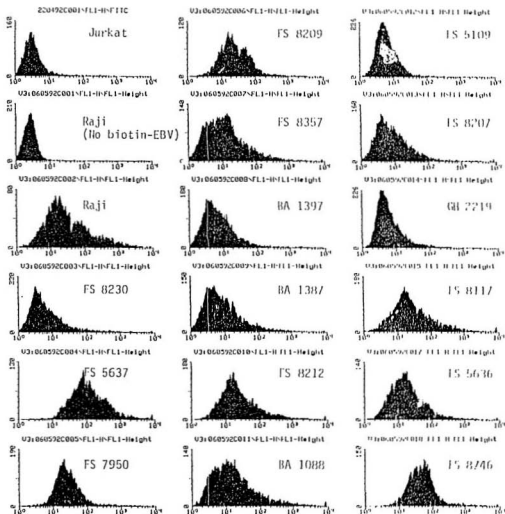


Figure 6.14. Flow cytometric profiles of cell lines reacted with biotin-EBV and stained with FITC-avidin. Non-reacted Raji is included as a negative control.

cells were incubated with the inhibitory (refers to inhibiting binding of EBV and C3d) anti-CR2 monoclonal antibody OKB-7 either before or after addition of biotin-EBV. In the second pair of tests, the conditions were the same, except for use of HB-5, a non-inhibitory anti-CR2 monoclonal antibody instead of OKB-7. In the third pair of tests, cells were either incubated with biotin-EBV, or with biotin-EBV pretreated with monoclonal antibody 72A1 (which is specific for gp350/220, the EBV ligand for CR2). The results of these tests are shown in Fig. 6.15. Under non-inhibiting conditions (line 1), approximately 40% of the cells of the FS 8212 line bound biotin-EBV. When cells were pre-incubated with anti-CR2 monoclonal antibody of either HB-5 (line 3) or OKB-7 (line 4) prior to the addition of biotin-EBV to cells, the percentages of positive cells were reduced by about a quarter, down to about 30%. When anti-CR2 antibody OKB-7 (line 2) was added after the biotin-EBV pre-incubation of cells, the percentage of positive cells was unchanged. Furthermore, the EBV-neutralizing Mab 72A1, which specifically binds to the gp350/220 of EBV, did not show complete inhibition of biotin-EBV binding to CR2. The inhibition was incomplete irrespective of whether the antibody was added after incubation of cells with biotin-EBV (line 5), or the biotin-EBV was first neutralized with the antibody and then added to the cells (line 6).

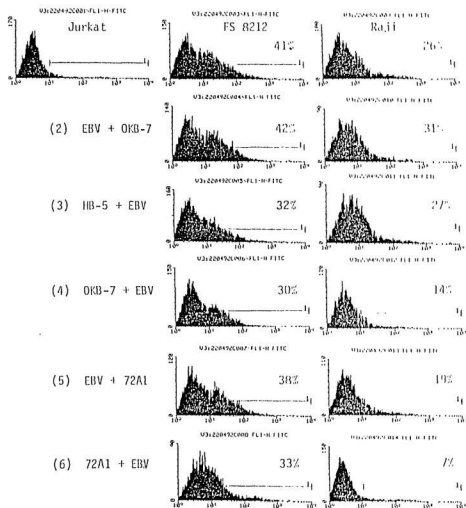


Figure 6.15. Flow cytometric profiles of cell lines reacted with biotin-EBV before or after treatment with mAb to human CR2. Line 1 shows binding of biotin-EBV to the three cell lines, Jurkat, FS 8212 (L/L phenotype) and Raji (H/H phenotype). In line 2, cell lines FS 8212 and Raji were first reacted with biotin-EBV followed by reaction with mAb OKB-7 (see text for further details).

In contrast, with the reference cell line Raji, about 26% of the cells were positive under non-inhibiting conditions. When the antibody OKB-7 was added after the biotin-EBV pre-incubation of cells, the percentage of positive cells was unchanged (the actual reading was 31%). However, when the cells were incubated first with OKB-7 and then with biotin-EBV, the percentage of positive cells dropped down to 14%. The lowest binding of EBV was found when biotin-EBV was pre-neutralized with mAb 72A1.

In summary, the cell, FS 8212 carrying the variant CR2 molecule, has not lost its EBV binding capacity. There are at least two explanations. The partial inhibition of EBV binding by OKB-7 mAb might indicate conformational changes of the variant CR2 so that it still can bind to EBV and/or C3d ligands. Unfortunately, an attempt to do the C3d binding assay failed due to technical problems. On the other hand, the data might suggest that another molecule rather than CR2 serves as a receptor for EBV on these cells.

6.5. The immunoregulatory functions of CR2

Animal models were established to investigate the role of CR2 in the regulation of immune responses. By following

different protocols, the primary and secondary antibody responses in vivo could be evaluated. These experiments are presented in the following two sections.

6.5.1. The influence of CR2 on the primary antibody responses

The protocol for the following experiments is illustrated in Fig. 6.16 and described in Materials and Methods.

Suppression of primary response IgM but not IgG by mAb to CR2 (7G6): As shown in Fig. 6.17, the first experiment included the 7G6-treated groups and the PBS-treated control groups, with four mice in each group. Mice treated with 200 ug of 7G6 mAb in 0.2 ml PBS or 0.2 ml PBS alone were immunized 24 h later with 100 or 200 ug of Ag. IgM and IgG responses were tested from day 3 (72 h) to day 18. In mice pretreated with PBS only, IgM and IgG peaked on day 5 and day 8, respectively. Compared to these, the 7G6-treated mice had substantially depressed IgM responses but nearly normal IgG responses, irrespective of the dose of Ag used. Based on student's *t* test, highly significant differences between the 7G6-treated group and the control group were found in the IgM response from day 3 to day 11, especially when 100 ug of Ag

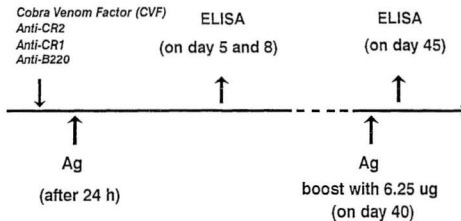


Figure 6.16. The protocol for evaluation of CR influence on the primary antibody response.

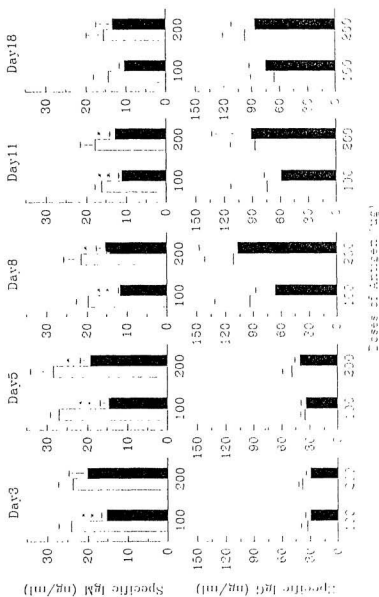


Figure 1. Specific IgM and IgG antibody levels in mice infected with 750 m.i.d. units. Mice were infected with 750 m.i.d. units of virus and bled at 3, 5, 8, 11, and 18 days post-infection. Serum was assayed for specific IgM and IgG antibody levels. The results are expressed as the mean \pm standard deviation of four mice per group. * indicates a significant difference ($p < 0.05$) between the infected and control groups.

were used. In contrast, no significant differences were found between the two groups in the IgG response.

Suppression of primary antibody responses by CVF vs mAb to CR2: In the next experiment, mAbs 7G6, RA3-6B2 as well as CVF were tested in parallel. The mAb RA3-6B2 reacts with the B cell specific molecule B220, and served as a control (Fig. 6.18). Suppression was again seen here, 7G6 inhibited IgM (day 5) but not IgG. In contrast, the control mAb (RA3-6B2) did not depress either the IgM or the IgG responses. The suppression of IgM production by 7G6 was highly significant, according to evaluation by one-way ANOVA. If the present experiment is considered a repeated test for confirmation of the conclusion from the previous experiment, student's one-tailed t test (7G6 treated group versus PBS treated group) still shows a significant difference on day 5.

These preliminary results as well as data published by other investigators have demonstrated that the primary antibody responses to a variety of T-dependent antigens can be depressed by both CVF and mAb to CR2. A direct comparison of the effects of these two reagents is made here to address the question of whether they do in fact have similar effects, a question which has not been addressed in previous

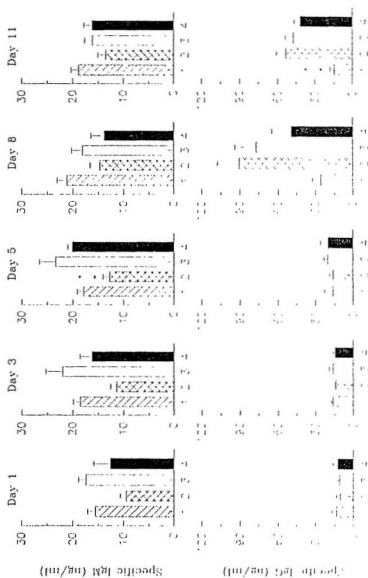
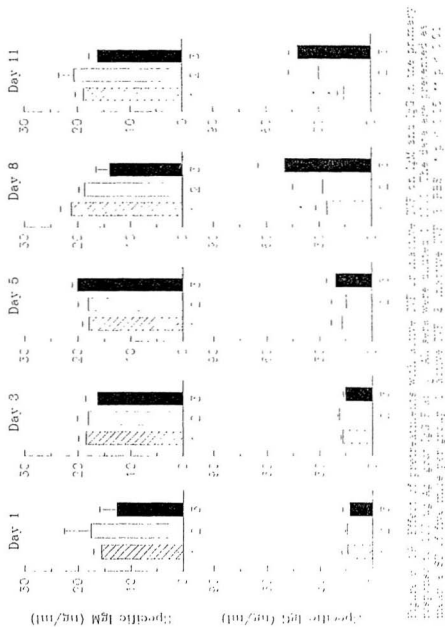


Figure 1. Specific IgM and IgG responses in BALB/c mice 127 in the primary response to BSA and Ad. The data are presented as mean \pm SD of five mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001. Ad, Ad; Ad + BSA, Ad + BSA; Ad + BSA + BSA, Ad + BSA + BSA; Ad + BSA + BSA + BSA, Ad + BSA + BSA + BSA.

publications. Surprisingly, CVF suppressed IgG only whereas mAb to CR2 (7G6) suppressed IgM (Fig. 6.18). Suppression of IgM and IgG was followed throughout the entire experiment (from day 1 to 11) without any changes, i.e. the IgM response in 7G6 treated mice remained suppressed from day 1, the IgG response from day 8 in CVF treated mice. The inactive CVF did not suppress either IgM or IgG (Fig. 6.19), when the three groups (Inactivated CVF-treated mice, CVF-treated mice and PBS-treated mice) were compared. Effects of different monoclonal antibodies against CR1/CR2 were also compared. Both 7G6 and 7E9 recognize CR1 as well as CR2 while 8C12 is monospecific for CR1. As shown in Fig. 6.20, IgM production can be depressed by either 7G6 or 7E9, but not 8C12. In addition, 7E9 also inhibits the IgG response while 7G6 in this experiment actually enhanced the IgG response. The precise binding sites of 7G6 and 7E9 on CR1/CR2 are unknown.

Effect of pretreatment with 7G6 on secondary responses:

Forty days after the primary response, the mice were boosted with Ag to see if pretreatment with CVF or mAb to CR1/CR2 had a long term effect on secondary antibody responses. Under normal circumstances optimal secondary antibody responses can be achieved with a dose of 6.25 ug Ag (as will be shown later, Fig. 6. 26). In the present experiment each primed mouse was



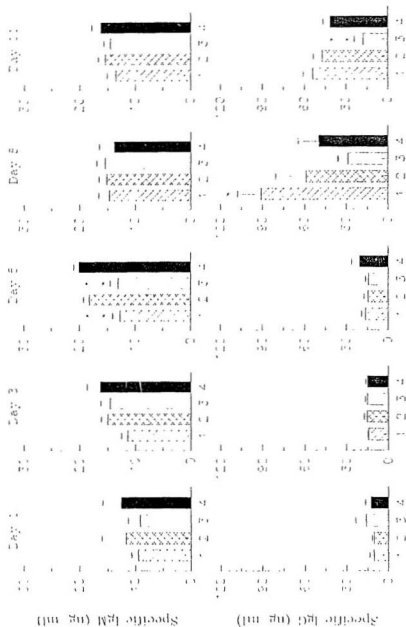


Figure 6.20 Effect of pretreatments with three different mAb to mouse CR1/CR2 on IgM and IgG in the primary response to 100 ug Ag (goat IgG F(ab')₂). All sera were diluted 1/1000. The data are presented as mean \pm SD of five mice per group, except group 1 which contains four mice. 1: mAb 7G6; 2: mAb BC12; 3: mAb 7E9 + PBS; * p < 0.05; ** p < 0.01.

given 6.25 ug free Ag in PBS and bled five days later for ELISA tests. The data in Fig. 6.21 suggest that the 7G6- and 7E9-treated mice had impaired secondary responses. As shown earlier (Fig. 6.19), 7E9 mAb suppressed both primary IgM and primary IgG production. From these experiments it may be concluded that pretreatment of mice with anti-CR2 mAb may lead to an impaired generation of immunological memory. On the other hand, CVF, regardless of whether it is given in the active or inactive form, has little influence on the secondary antibody response. Anti-CR1 (mAb 8C12) also has minimal effect on the secondary response.

Influence of pretreatment with CVF or mAb to CR on IgG subclass responses: To further assess precise changes, IgG subtypes in the secondary antibody responses have been determined. Pretreatment with mAb to CR2 suppressed IgG_{2a} and IgG_{2b} but had no or little effect on IgG₁ and IgG₃ (Fig. 6.22). Compared to the control group (PBS-treated), mice pretreated with RA3-6B2 or CVF (active or inactive) showed no notable changes in any of the IgG subclasses.

Effect of anti-CR2 treatment on established immunological memory: As described above, mice treated with mAb to CR (particularly, 7G6 mAb to CR2) 24 h prior to immunization

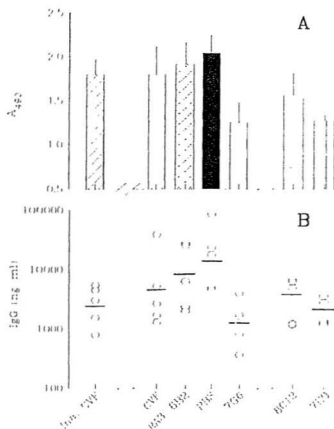


Figure 6.21. Effect of pretreatments on secondary IgG response to Ag given at an optimal dose of 6.25 μ g on day 40. The data are presented as mean \pm SD of five mice per group, except the 7GG treated group containing four mice. The animals were bled 5 days after being boosted. All sera were diluted 1/1000. (A) the optical values (arithmetic means); (B) the concentrations of specific IgG (geometric means).

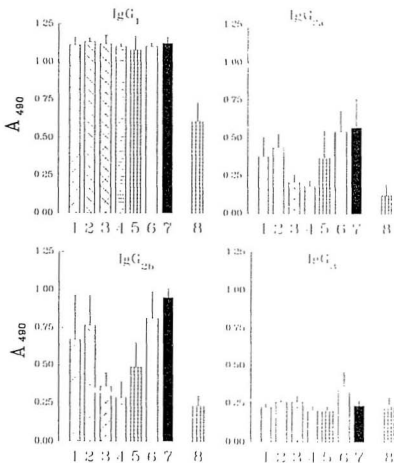


Figure 6.22. IgG subclass of specific antibody in the secondary response among the pretreated groups boosted on day 40. All sera were diluted 1/1000. The data are presented as mean \pm SD of five mice per group, except group 8 containing only four mice. 1, inactive CVE, 2, active CVE, 3, mAb 7G6, 4, mAb 8C12, 5, mAb 7E9, 6, mAb RA3-6H2, 7, PBS, 8, group 7 before boost (on day 40). The animals were bled 5 days after being boosted.

showed both diminished IgM primary response and impaired immunological memory. Once mice had developed normal primary and secondary responses, however, their immunological memory was not affected by treatment with mAb to CR2. Four groups of mice were boosted with Ag on day 180. They had been injected with mAb or PBS on the previous day (day 179). As shown in Fig. 6.23, all mice, irrespective of treatment, produced the same amount of specific antibody.

6.5.2. Role of CR2 in the secondary antibody response

The protocol for the following experiments is illustrated in Fig. 6.24 and described in Materials and Methods. Here, the antigen is targeted by mAb to the B cell or the follicular dendritic cell.

Kinetics of primary antibody response: A group of four mice was immunized with 200 ug of Ag per mouse and the mice were bled at different times afterwards, from day 3 to day 18. IgM and IgG responses were monitored by ELISA. As before (Fig. 6.17), IgM peaked on day 5 and IgG peaked on day 8, then both gradually declined. By day 18 the level of specific IgG appeared close to the normal range of IgG, which is indicated by upper and lower dashed lines (Fig. 6.25).

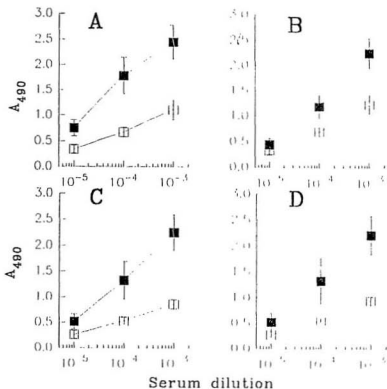


Figure 6.23. Effect of pretreatment with mAb 7G6 on an established immunological memory. Mice were injected with Ag on day 0 and 11. 180 days later they were treated with 200 μ g mAb (7G6 or RA3-6B2) or PBS alone. 24 h later, the mice were boosted with Ag again. IgG was tested 5 days later. (■) before boost, (□) after boost. (A) 7G6-treated, boosted with 6.25 μ g Ag, (B) 7G6-treated, boosted with 6.25 μ g Ag, (C) RA3-6B2 treated, boosted with 6.25 μ g Ag, (D) PBS-treated, boosted with 6.25 μ g Ag. The data are presented as mean \pm SD of four mice per group. Each sample was tested in three dilutions.

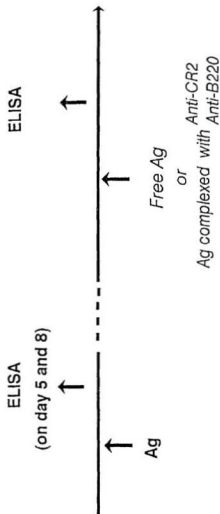


Figure 6.24. The protocol for evaluation of the role of CR2 in the secondary antibody response.

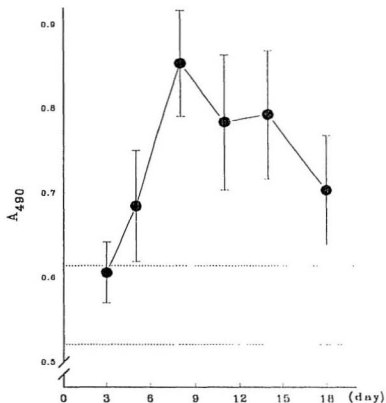


Figure 6.25. Time-course analysis of the primary specific IgG response to antigen (200 ug of goat IgG F(ab')₂). All sera used for ELISA were diluted 1/1000. The results are presented as mean ± SD of four mice per group. Upper and lower dashed lines denote the 95% confidence interval of the unimmunized controls.

Secondary antibody responses to different doses of Ag: To determine the amount of Ag needed for induction of a secondary antibody response, mice were immunized initially with 200 ug of Ag per mouse, then boosted with decreasing doses of Ag, from 100 ug to 0.0625 ug. Four days later, the mice were bled and the secondary antibody response examined (Fig. 6.26). It was found that the secondary antibody response could be induced by a minimum of 6.25 ug Ag. Higher doses did not substantially increase the response, and secondary responses were poor when less than 6.25 ug of Ag was given. Thus, in this case 6.25 ug of Ag was the minimal dose required for the secondary response.

Efficiency of Ag complexed with anti-CR2 mAb for induction of secondary response: In these experiments the antigen was targeted to the B cell or follicular dendritic cell with mAb to evaluate CR2 function in the secondary immune response. Two separate experiments were conducted for boosting the primed mice with different doses of complexes. One was undertaken on day 14, similar to that described above for free antigen. The other was done on day 40 when measurable amounts of specific antibodies from the primary response had disappeared. The

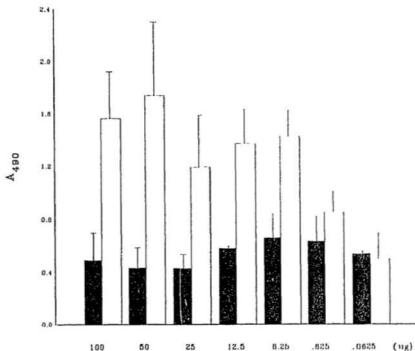


Figure 6.26. The effect of antigen dose on secondary response. The antigen was administered on day 14 and the antibody detected by ELISA on day 18. (■) before boost and (□) after boost. Each bar represents mean \pm SD of four mice per group.

results are presented by optical density values, as mean \pm SD for each group for the first experiment (Fig. 6.27) and as individual data for each animal for the second experiment (Fig. 6.28). The amount of specific antibody in ng/ml was also calculated for the second experiment (Table 6.5). These experiments both showed that the secondary antibody responses were enhanced when the antigen was complexed with anti-CR2 mAb (7G6), particularly when trace amounts of antigen (0.0625 ug) were used. There was no secondary response to the antigen at this low dose when given as uncomplexed antigen (Fig. 6.26). When anti-B220 mAb (RA3-6B2) was used, the secondary responses were only modestly improved. To demonstrate that the secondary antibody response was specific for Ag (goat IgG F(ab')₂ fragment), the antibody responses to rat IgG (mAbs 7G6 and RA3-6B2) were simultaneously monitored by ELISA. These responses were primary responses (IgM) and specific mouse IgG antibody to rat was very low (data not shown).

Specific IgG subclasses were also tested for the second experiment. Apparently, the elevated antibody response was conferred by more production of IgG_{2a} and IgG_{2b}, as indicated in Fig. 6.29. IgG₁ and IgG₃ levels seemed not to be affected.

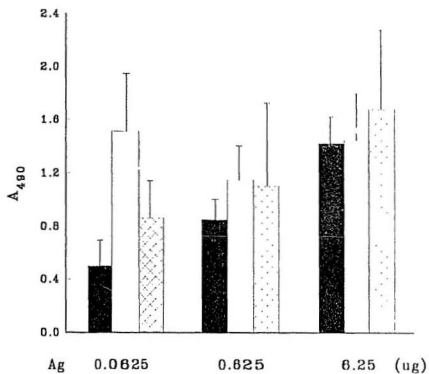


Figure 6.27. Secondary response to different doses of complexed antigen on day 14 (Exp. 1). All sera were diluted 1/1000. (■) Ag alone; (□) Ag + 7G6 (anti-CR2); (▨) Ag + RA1-6B2 (anti-B220). The data present mean \pm SD of three mice per group.

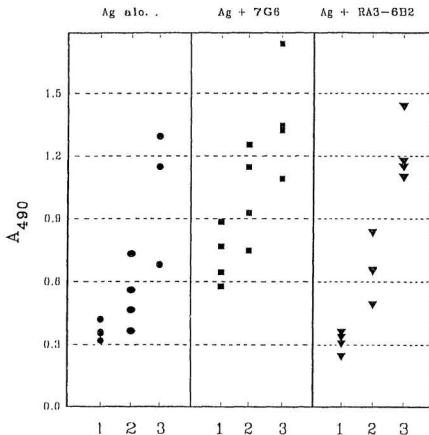


Figure 6.28. Secondary response to different doses of complexed antigen on day 40 (Exp. 2). All sera were diluted 1/1000. (1) 0.0625 ug Ag; (2) 0.625 ug Ag; (3) 6.25 ug Ag. The data are reported as spots representing individual mice. The animals were bled 4 days after being boosted.

Table 6.5. Quantification of specific IgG by ELISA.

Ag (ug)	Ag form	\log_{10} IgG concentration	Mean* (ng/ml)	P **
0.0625	Ag alone	1.39 ± 0.08	24.70	
	Ag + 7G6	2.35 ± 0.33	225.26	<.01
	Ag + RA3-6B2	1.46 ± 0.12	28.80	NS
0.625	Ag alone	1.84 ± 0.30	68.83	
	Ag + 7G6	3.35 ± 0.41	2257.87	<.01
	Ag + RA3-6B2	1.82 ± 0.49	66.34	NS
6.25	Ag alone†	3.18 ± 0.79	1525.11	
	Ag + 7G6	3.64 ± 0.31	4382.27	NS
	Ag + RA3-6B2	3.35 ± 0.20	2245.43	NS

The sandwich ELISA was performed, as described in Materials and Methods.

* Geometric mean (antilog of the \log_{10} IgG concentration).

** determined by one-way ANOVA, and then compared with the group of Ag alone, if necessary.

† three mice in this group.

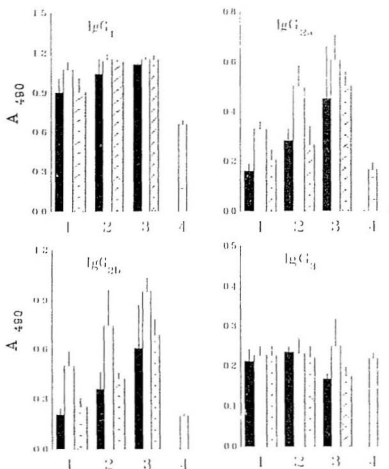


Figure 6.29. IgG subclasses in the secondary response to different doses of complexed antigen on day 40 (Exp. 2). All sera were diluted 1/1000. (■) Ag alone; (□) Ag + 7G6 (anti-CR2); (▨) Ag + RA3-6D2 (anti-B220). The data present mean \pm SD of each group. (1) 0.0625 ug Ag; (2) 0.625 ug Ag; (3) 6.25 ug Ag. (4) IgG subclass levels on day 40 (before boost).

CHAPTER 7

DISCUSSION

7.1. Factor H typing

The study of genetic polymorphisms of proteins is usually carried out through analysis of size, charge and antigenic determinants. The search for charge differences is classically done by electrophoresis or isoelectric focusing of serum/plasma samples followed by the identification of the particular protein. These techniques have been fruitfully applied to the study of polymorphisms of complement proteins, and almost all the proteins of the complement system have been found to be polymorphic. Functional differences have been suggested or described for some components, such as C4A versus C4B (Law et al., 1984).

Factor H is a single-chain glycoprotein with a molecular weight of 150,000 daltons in plasma. It belongs to the family of complement activation regulators, and functions both to accelerate the decay of alternative-pathway C3 convertase (C3bBb) and as a cofactor for factor I in the breakdown of C3b to iC3b. Polymorphism of factor H was first described in 1984.

So far, five genetic variants (FH1-FH5) which differ in isoelectric point have been identified (Rodriguez de Cordoba and Rubinstein, 1984, 1987).

The present study was carried out with the purpose of simplifying the typing procedure and establishing factor H allele frequencies for the Newfoundland population. The protocol described by Rodriguez de Cordoba had some experimental disadvantages. For example, for immunoprecipitation, a considerable amount of anti-human factor H antiserum (at least 100 ul) was needed for each sample. Sometimes such a requirement for a routine test is impractical and uneconomical. These limitations are obstacles to human population studies.

The approach presented here employed the sensitive immunoblotting technique. Amounts of less than 5 ul human serum were enough for the test, and the experimental costs in time and antibody were much lower. This method meets the general requirements for a population survey, and may easily be adopted by other laboratories.

The reliability of the revised method was verified by Dr. Rodriguez de Cordoba, who, by means of his original method,

re-typed a small group of samples with phenotypes defined in our laboratory, confirming that the data obtained by these two methods were consistent with each other. By typing a group of 129 local unrelated individuals, we found that the frequencies for two common alleles, *FH*1* and *FH*2*, in our population (Zhou and Larsen, 1990) and the data previously reported for New York City, were comparable (Rodriguez de Cordoba and Rubinstein, 1984). There were no rare variants of factor H identified in this study.

A possible association between factor H variants and susceptibility to rheumatoid arthritis was also tested. Although some HLA molecules are strongly associated with susceptibility to this autoimmune disease, the actual pathogenesis is still largely unknown. It has been suggested that early complement components may participate in and/or accelerate the course of autoimmune diseases since these components play a role in the clearance of immune complexes, the generation of immunoregulatory factors, immune complex solubilisation, and viral neutralization. Therefore, factor H, as a regulator of C3 convertase, might be associated with one or more autoimmune diseases. This might suggest that there were functional difference between the factor H variants. The data, obtained from 111 patients with rheumatoid arthritis in

this study, did not reveal an association between the disease and any factor H alleles.

In a more recent report, an alternative method for factor H typing has been described by Nakamura et al (1990), who used a horizontal, ultra thin-layer polyacrylamide slab gel for isoelectric focusing of factor H in serum. They found that there were three common phenotypes of factor H and five rare patterns in Japanese. The existence of a null allele was also suggested. Family studies confirmed that this factor H polymorphism is controlled by two common alleles. However, there was no correspondence between the common alleles defined by this technique and those so far reported by Dr. Rodriguez de Cordoba and in our study. This raises the question of whether there are two polymorphic systems of factor H.

As mentioned earlier (in CHAPTER 2), human factor H does appear in three forms in plasma. The 150,000 dalton molecule is the common one; the 47,000 and 43,000 dalton molecules are truncated forms, produced as a consequence of alternative mRNA splicing of the common transcript. It is not known whether these two truncated forms are also polymorphic. Obviously, more studies of different populations are required and the relation between the two polymorphic systems must be

clarified. Finally, the nomenclature for factor H variants should be unified in the next Complement Genetics Workshop.

7.2. Factor I typing

Factor I is a regulatory enzyme which cleaves and inactivates C3b and C4b in the presence of cofactors. Genetic polymorphism of factor I was first demonstrated in a Japanese population (Nakamura and Abe, 1985). Two common alleles, *IF**A and *IF**B, were identified.

The original purpose of this study was to explore the factor I phenotypes in a Caucasian population. A large group of samples from the local Caucasian community was screened. Surprisingly, an identical pattern was observed in all the samples tested. It was initially suspected that the method employed was inappropriate. However, when a panel of reference samples from Drs. Nakamura and Yuasa were included on the gel side by side with test samples, the reference samples did show the expected patterns. Sera from a small group of local Chinese donors were then tested.

In total, 305 individual samples (288 Caucasian and 17 Chinese) were typed for factor I. No *IF* A phenotype was

observed in the Caucasian samples. However, a new variant was identified, and confirmed when the family was typed (Zhou and Larsen, 1989). In contrast, of the 17 Chinese individuals tested 7 were IF AB heterozygous. Thus, it appears that the IF*A allele, which occurs quite frequently in some Asian populations, seems to be present in Caucasian populations at a very low frequency if at all. This phenomenon was also noticed a few months earlier by Yuasa et al (1988), who studied factor I in a French population. A geographical distribution of the IF*A allele is shown in Fig. 7.1 using data from several reports. The combined data suggest the existence of a geographical cline for allele IF*A from Europe to Japan. The Nepalese are characterized by an intermediate value between the French and the Japanese, reflecting a point of contact between Mongoloids and Caucasoids. These findings suggest that factor I may be useful for anthropogenetic studies, and further investigations in other ethnic groups (such as Amerindians and Australian aborigines) would be of interest.

In the Vth Complement Genetics Workshop, factor I reference typing was examined, and the nomenclature for factor I allotypes was officially designated according to the direction of each variant's pI relative to two common types.

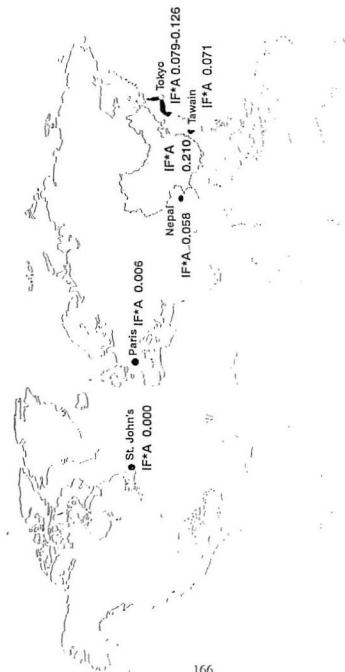


Figure 7.1 Geographical distribution of IF* A allele.
 (Based on our data and other reports of Nakamura et al.,
 1985, Nishimukai et al., 1985 and Yuasa et al., 1988)

So far, four variants have been found and named, including the two common ones IF A and IF B, and two rare variants IF A1 (Nakamura et al., 1990) and IF B1 (defined in this study).

7.3. CR2 typing

Human CR2 is a 145 kDa integral membrane glycoprotein. The primary ligand for CR2 is C3d, a processed form of C3 which results from factor I-mediated proteolytic cleavage of C3b. CR2 also serves as the Epstein-Barr virus receptor and probably the receptor for interferon- α (Delcayre et al., 1991). As a member of the family of Regulators of Complement Activation, the CR2 molecule contains multiple copies of the 60 amino acid short consensus repeat (SCR). Recent experiments have shown that the gene encoding CR2 is within a region on chromosome 1q³² which also contains the genes for CR1, MCP, DAF, C4BP and factor H (Weis et al., 1987; Lublin et al., 1987, 1988; Hing et al., 1988). Polymorphic variants have been described for all of these proteins with the exception of CR2 (Hourcade et al., 1990).

Even though CR2 and CR1 evolved via the same mechanism of expansion of short consensus repeats (SCRs), there have not been any variant forms of the CR2 protein identified that are

analogous to the polymorphic gene products of CR1. However, a number of studies strongly suggest the existence of polymorphism in the CR2 protein at the structural level. The first evidence came from the characterization of two forms of CR2 cDNA isolated from a single tonsillar library (Weis et al., 1987). Further studies on the sequence of the variant cDNA clone led to the identification of several nucleotide changes, indicating changes in the amino acid sequence of the protein (Moore et al., 1987; Toothaker et al., 1989). These changes may reflect different CR2 alleles existing in the human population. Further evidence came directly from the study of RFLPs of the CR2 gene, using Taq I or Hae III restriction enzymes (Fujisaku et al., 1989).

The present study demonstrated, for the first time, polymorphism of human CR2 at the protein level. The CR2 molecule of 145,000 daltons which is the common product of the CR2 gene, co-exists in the population with a variant CR2 molecule of about 75,000 daltons. This observation was based on SDS-PAGE analysis of EBV-transformed B cells from different individuals. The two forms of CR2 protein were tentatively designated H and L, representing CR2 variants with high (145 kDa) and low (75 kDa) molecular weight, respectively. The pattern of inheritance of the H and L variants was consistent

with autosomal codominant expression of two alleles, based on family data where the father and the daughter are H/L heterozygous but the mother and the son H/H homozygous. A homozygous individual of L/L type as well as six heterozygous individuals were also found within the group of 63 samples tested. The observed frequencies of three phenotypes did not differ from those predicted by Hardy-Weinberg equilibrium based on gene frequencies of 0.937 and 0.063 for CR2 H and CR2 L, respectively (Table 6.4). Although actual allele frequencies of CR2 in a large population may vary slightly, the fairly high frequency of the variant L allele suggests that it is not a very rare allele and that this is a true polymorphism.

The mechanism by which the variant L protein was produced is likely to be similar to that of the generation of CR1 variants. The variant L protein might contain only two LHR-like segments, since its molecular weight was half of that of the common H variant composed of four LHR segments. Such speculation, of course, must be confirmed by studies of genomic DNA and/or cDNA sequences. This is beyond the objective of the present project. In addition, functional studies of the variant L protein may provide some new information about the active domains of the CR2 molecule.

As indicated in Fig. 6.10, the immunoprecipitate of CR2 from B cell lysates from some, but not all, individuals also yielded an extra band with lower molecular weight than CR2 L on SDS-PAGE. The molecular weight of this extra band was approximately 50 daltons. This extra band may originate from one of several sources. Since B cell surface Ig molecules are present in the cell lysates, the binding of CR2-anti-CR2 complexes to Protein-A could be contaminated with human sIg molecules. This is unlikely, since all the cell lysates had been pre-cleared with Protein-A beads prior to CR2 precipitation and, even if the pre-clearing was not complete, the extra band was not observed in all the samples. An alternative explanation for the extra band derives from the structure of the CR2 complex and its components. It is known that human CR2 is usually associated with another cell differentiation antigen, CD19 (Matsumoto et al., 1991), as well as with CR1 (Tuveson et al., 1991), on B cells. In the studies of Matsumoto et al. (1991), monoclonal antibodies to CR2 could co-precipitate the CR2-CD19 complex from digitonin lysates of Raji B lymphoblastoid cells. Three unidentified components: p130, p40 and p20, were also co-precipitated. Anti-CD19 precipitated four of these components, corresponding to CR2, CD19, p130 and p20, but not p40. In the presence of the nonionic detergent NP-40, anti-CR2 precipitated only three

proteins, CR2, p130, and p40 whereas anti-CD19 precipitated CD19 and p40, but not CR2, p130 and p20. Thus, p40 seems to be tightly associated with both CR2 and CD19, depending on the choice of detergent used. Therefore, it is likely that the extra band presented here is p40, although other explanations have not been excluded (such as proteolysis of CR2 or alternative splicing of CR2 mRNA). Variation of radioactive density of the extra band among the different cell lines may be the result of varying molar ratios of CR2 and p40. The slight difference in molecular mass between this newly identified extra band and p40 could be due to the conditions of SDS-PAGE analysis. The gel concentration used in this laboratory was 7.5% while a 5-15% gradient gel was employed in the studies of Matsumoto et al. (1991).

7.4. Characterization of epitopes on CR2 variants by flow cytometry.

The relationship between structure and function in the variant L molecule was approached by flow cytometry. The primary structure of human CR2 has been deduced from sequence analysis of the cDNA (Weis et al., 1987; Moore et al., 1987). Its extracytoplasmic domain is composed entirely of 16 (or 15) short consensus repeats of 60 amino acids that are typical of

the Regulators of Complement Activation. Using a panel of synthetic peptides corresponding to the sequences of the CR2 ligands, recombinant CR2 mutants, and monoclonal antibodies to CR2, the epitopes on human CR2 specific for ligand binding have been precisely defined (Lowell et al., 1989; Carel et al., 1990). The binding sites for both C3d and Epstein-Barr virus are mapped within the same or two very close segments, spanning the first two SCRs of the molecule.

Analyses of the binding specificity of the CR2 variant molecule might yield information on a possible alteration in function caused by change in molecular size. By means of biotin-labelled EBV particles, flow cytometry of the binding between EBV and CR2 was carried out using different cell lines. In general, expression of CR2 on the cell surface was found to vary tremendously from one cell line to another. This is very similar to variation observed for CR1 expression. There were several monoclonal antibodies available for testing the specific epitopes of the CR2 molecule (Lowell et al., 1989; Carel et al., 1990). OKB-7 can block both C3d and EBV binding site(s) on SCRs 1-2 of CR2; neither HB-5, which recognizes SCRs 3-4, nor B2, specific for SCRs 9-14, can do so. The data presented here showed that the CR2 variant L molecule had retained its epitope for EBV binding, indicating

that its function in terms of reaction with this ligand was unchanged in spite of the reduced molecular mass.

Monoclonal antibodies to SCRs 1-2 (OKB-7), 3-4 (HB-5) and 9-14 (B2) all bind the variant CR2, suggesting that all these SCRs spanning "LHR-like" 1, 3 and at least part of 4 of the CR2 molecule are present on the variant molecule. The mechanism responsible for the reduction of molecular mass is unknown. Based on the positive reaction with mAbs OKB-7, HB-5 and B2, it would appear that SCRs 1-2, 3-4 and 9-14 are present. This leaves SCRs 5-8 and 15-16 unaccounted for. However, it would be surprising to find two non-consecutive sequences of the gene either lost or not translated. It is possible that there could be some alteration in post-translational modification, such as glycosylation, on the smaller CR2 L molecule, leading to the differential binding of antibodies. The present study raises some doubts about the defined location of the B2 binding site. Two previous reports arrived at different conclusions. One mapped the B2 epitope to SCRs 9-11 (Carel et al., 1990) whereas another localized it to SCRs 11-14 (Lowell et al., 1989). Therefore, DNA and mRNA studies may be more useful to clarify the molecular basis for the loss in size. Second, since the homozygous L/L cell line was originally derived from peripheral B cells by EBV-

transformation and because OKB-7 could only partially block EBV binding to CR2, it is possible that there is a second receptor independent of CR2 to mediate the virus infection of these cells. The finding of this homozygous L/L cell line may provide a unique opportunity to study the mechanism of EBV tropism.

7.5. Summary

Genetic polymorphisms of factor H, I and CR2 were investigated by a combination of isoelectrofocusing and immunoblotting or by SDS-PAGE. The new method for factor H is reliable and economical, and meets the general requirements for a population survey. The allelic frequencies in the Newfoundland population were estimated from 129 unrelated individuals to be 0.597 and 0.403 for *FH*1* and *FH*2*, respectively. No association of factor H types with rheumatoid arthritis was found in 111 patients tested.

288 Caucasian individuals were typed for factor I, and a new variant, *IF B1*, was found. The phenotype *IF AB* is lacking in the population studied, indicating that the *IF*A* allele may be present in Caucasian populations at a very low frequency. It may occur quite frequently in Asiatic populations.

For the first time, CR2 protein polymorphism was demonstrated. There are two variants, CR2 H (heavy) and CR2 L (light). In 63 samples there were six heterozygotes and one homozygote for CR2 L. The inheritance of the *CR2*L* allele was confirmed in a family study. The CR2 L variant of 75 kDa may represent the product of an allele varying in the number of short consensus repeats. Flow cytometry analysis of the ligand/epitopes on CR2 L did not clarify the molecular basis for the loss in size.

7.6. CR2 immunoregulatory functions

That the complement system is important for humans and animals in mounting a normal antibody response has been demonstrated in a number of different ways. Treatment of mice with cobra venom factor (CVF) leads to complement depletion by activating the alternative pathway and suppresses the antibody response to both T-dependent and T-independent antigens (Pepys, 1976). Hereditary deficiencies in complement components C2, C3 and C4, described in man, guinea pigs, and dogs, generally lead to a depressed antibody response and poor induction of immunological memory (Bottger et al., 1985; O'Neil et al., 1988; Jackson et al., 1979; Ochs et al., 1983). These data imply that the presence of C3, a central element

for both complement activation pathways, is critical for induction of antibody responses, and that the impact of the C3-activating factors C2 or C4 is probably secondary to the actual effect of C3.

Characterization of complement receptors has added to our understanding of C3 function in immune responses. In humans, CR1 is expressed on a variety of lymphoid cells. CR2 expression is mainly restricted to B cells although follicular dendritic cells, thymocytes and a small number of T cells have also been shown to be CR2-positive (see p. 50). The role of these receptors in immunoregulation has been demonstrated by a number of experiments. For instance, monoclonal anti-CR antibodies (particularly anti-CR2) induce human B cells to proliferate and to secrete Ig in vitro (Wilson et al., 1985; Nemerow et al., 1985), and aggregated C3b or C3d act as growth and differentiation factors affecting pre-activated murine B cells (Erdei et al., 1985; Malchers et al., 1985).

Studies of mouse complement receptors provided a means of further determining the roles of CR1 and CR2. There is approximately 70% identity between the predicted amino acid sequence of human CR2 and the murine homologue (Fingeroth et al., 1989; Fingeroth, 1990). Both have the same molecular

weight, containing 15 SCRs. Murine CR1 (MCR1) is a product of alternatively spliced mRNA of the CR2 gene. Hence, among the 21 SCRs of the extracellular domain of MCR1 there are only 6 unique CR1-like SCRs (Kurtz et al., 1990; Molina et al., 1990). Similarities in structure between human CR2 and MCR2/MCR1 make mice an appropriate experimental model in which to study the function of complement receptors in vivo. Here, a series of studies were carried out to explore the role of complement receptors in the regulation of antibody responses and in the induction of immunological memory.

7.6.1. Regulation of the primary antibody response

The development of monoclonal anti-lymphocyte antibodies has greatly enhanced our ability to identify functionally distinct cells of the immune system and to manipulate these cells through their surface antigens. As a result, monoclonal antibodies have become valuable tools in studies of the cellular mechanisms underlying immune responses. The great advantage of monoclonal antibodies over antisera is their specificity for a single epitope of the antigen.

Previous studies have shown that mAbs to complement receptors were able to depress the primary responses to a

variety of T-dependent antigens such as horse erythrocytes (HRBC), sheep erythrocytes (SRBC), keyhole limpet hemocyanin (KLH) and bacterial antigen (*Salmonella*), and to a lesser extent, to T-independent antigens such as Ficoll and dextran (Heyman et al., 1990; Thyphronitis et al., 1991). The present studies have confirmed and extended the previous findings. Furthermore, a direct comparison has been made between the effects of CVF and mAb to CR2 in modulating humoral immune responses.

The monoclonal antibodies to mouse CR1/CR2 used here are three well-characterized rat IgG antibodies (Kinoshita et al., 1988, 1990; Molina et al., 1991). 7G6 (IgG_{2b}) is specific for murine CR2 but cross-reacts with CR1. 7E9 (IgG_{2a}) also reacts with murine CR2 as well as CR1. 8C12 (IgG_{2c}) is monospecific for murine CR1 and does not react with murine CR2. The anti-B220 monoclonal antibody, RA3-6B2 (IgG_{2b}) was used as a control mAb. The B220 molecule (CD45) is a cell surface marker for mature and immature B cells, with a developmental profile similar to that of CR2.

The results of this study can be summarized as follows: 1) Injection of anti-CR2 prior to injection of antigen leads to a poor primary IgM response, a normal primary IgG response,

and a poor secondary response when boosted 40 days later. 2) Injection of CVF prior to injection of antigen leads to a normal primary IgM response, a poor primary IgG response, and a normal secondary response when boosted 40 days later.

In order to explain these results, a scheme is presented to outline a series of events, some hypothetical, which may account for the experimental findings in this study. For the sake of simplicity, interleukins have not been considered here, assuming that neither CVF nor anti-CR2 affect the T cell responses. It is also assumed that crosslinking by Ag-Ab-C3b/C3d complexes is a requirement for a switch from IgM to IgG. (Fig. 7.2).

As shown on the left of this figure, PBS-treated mice developed a normal primary response, producing both IgM and IgG, and generating memory B cells. CVF-treated mice, indicated in the middle column, had a normal IgM response in the beginning, and memory B cells were generated. But, the switch to IgG induced by newly formed Ag-Ab-C3 complexes did not occur due to C3 deficiency. In mice treated with anti-CR2, shown on the right, it is hypothesized that induction of anergy led to decreased production of IgM and impaired generation of memory B cells. But, the primary IgG response

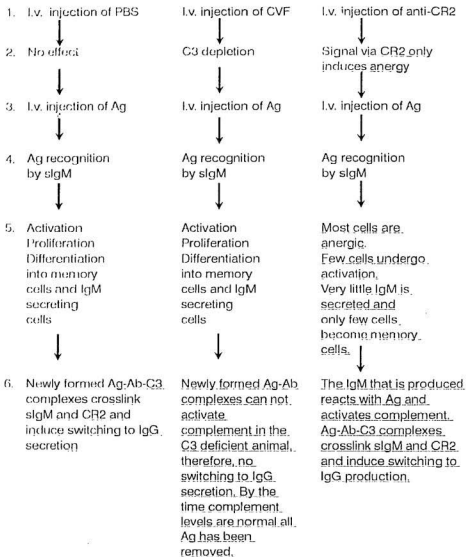


Figure 7.2. A series of events, some hypothetical (underlined), after pretreatments with PBS, CVF and anti-CR2 mAb, followed by antigen.

was normal, and Ag-Ab-C3 could induce the isotype switch. The hypothetical events indicated in the figure are discussed below.

The mechanism(s) by which mAb to CR2 suppress primary responses appear to be quite different to those of CVF. One difference may be the time of action. The interaction between CR and anti-CR mAb presumably takes place very quickly after a single injection of the mAb. In contrast, CVF was injected several times over 24 h. CVF is unlikely to react directly with the B cell. Instead, it probably first activates C3, and the C3-derived fragments bind to CR2 on the B cell. A second difference is that the mAb probably crosslinks CR2 receptors, while the monomer C3d fragments derived from activation of C3 by CVF bind to CR but are unlikely to crosslink them. Therefore, it is likely that pretreatment with anti-CR2 depresses IgM response because of B cell anergy induced by a single signal from CR2 without signalling via sIgM.

Finally, at a late stage in the anti-CR2 pretreated mice appropriate crosslinking of sIgM and CR2 via Ag-Ab-C3d may induce switching from IgM to IgG production. Such switching is unlikely to occur in the CVF-treated mouse which lacks C3. The reason is that, although pretreatment with CVF has little

influence on the IgM response, newly formed Ag-Ab may have been removed from circulation while the mouse is still complement deficient. One could test this hypothesis by injecting anti-CR2 a few days after Ag had been given in animals made C3 deficient by CVF. One would here expect normal IgG production.

That signalling via complement receptors somehow affects the switch from IgM to IgG₂ subclass, is also suggested by the results of two experiments (Fig. 6.22 and 6.29). A normal IgG₁ response is seen, but very little IgG_{2a} or IgG_{2b} is produced when animals, treated with anti-CR2 or anti-CR1 prior to the first exposure to antigen, are boosted with antigen 40 days later (Fig. 6.22). However, a normal IgG₁ response but an increased IgG_{2a} and IgG_{2b} response is seen in animals boosted with a suboptimal dose of Ag complexed with anti-CR2 (Fig. 6.29). This apparent association between CR signalling and IgG₂ production is worthy of further study. A previous study by Hebell et al. (1991) also showed that a primary immune response in mice to SRBC could be suppressed by a soluble recombinant CR2, and only IgG₁ subclass was unaffected. If the hypothesis that CR2 may preferentially influence class switching to IgG_{2a}/IgG_{2b} can be proven to be correct, it may provide one way of producing mAb of chosen subclass. We intend

to test this with a panel of IgM secreting hybridomas. A critical requirement of such experiments will be that the hybridomas are expressing membrane CR2.

This study differs from that of Heyman et al. (1990) and Thyphronitis et al. (1991), who found that both IgM and IgG responses were depressed in 7G6-treated mice. The discrepancy may be due to time of testing, or due to Ag and Ag dose used. The antigens used were HRBC and KLH (Heyman et al., 1990) or FITC-haptenated antigens (Thyphronitis et al., 1991). The ELISA for both IgM and IgG was performed 5 or 7 days later in both studies. In the present experiments, normal primary IgM responses could easily be detected by ELISA as early as day 3, with a peak on day 5. IgG responses predominated on day 7 or 8. Only IgM was profoundly depressed in anti-CR2 treated mice during the entire period of the primary response, although a higher dose (200 ug) of Ag had a slight compensatory effect. On the other hand, the IgG response was virtually unaffected irrespective of the amount of Ag used for immunization (Fig. 6.17).

The different specificities of these monoclonal antibodies may be responsible for their disparate effects. 7E9, like 7G6, suppressed IgM (Fig. 6.20), but unlike 7G6, it also depressed

the IgG response. This speculation that specific epitopes on CR2 affect the immune response differently gains indirect support from in vitro stimulation studies of human B cells. One mAb (OKB-7) induced B cell proliferation when used alone while another mAb (HB-5) activated B cells only in the presence of anti-mouse immunoglobulin antibody which crosslinks the HB-5 mAb (Nemerow et al., 1985). Anti-CR1 mAb 8C12 had no effect on either response. In addition, the results suggest that either the CR1 molecule or, at least, the 8C12 epitope within the first 6 MCR1-specific SCRs (Molina et al., 1992), has little or no impact on the primary immune response. This finding is consistent with a previous report by Heyman et al. (1990).

Another explanation for the differential effects of these monoclonal antibodies may be related to isotypes of the antibodies used. In general, rat IgG_{2b} antibodies more efficiently than IgG, deplete target cells in vivo as these cells circulate through the spleen (Seaman and Wofsy, 1988). However, this is unlikely to be the case in this study since Kinoshita et al. (1990) have shown that in vivo pretreatment with 7G6 did not markedly decrease the number of B cells, their size, or the expression of B cell sIgM, Ia, or B220 antigens.

7.6.2. Influence of CR2 on memory generation

Although both the anti-CR2 mAbs (7G6 and 7E9) and CVF suppressed the primary response, only pretreatment with anti-CR2 mAb affected the secondary antibody response. This finding is similar to that of a previous report by Wiersma et al. (1991). In addition, impairment of the secondary response to optimal doses of Ag was seen in 7G6-pretreated mice but not in mice treated with CVF, RA3-6B2 or PBS. This agrees with an earlier observation of Pepys (1974), who demonstrated that CVF treatment prior to a primary response had a stronger effect than the same treatment prior to a secondary response. It appears that a good primary IgM response was followed by the generation of memory B cells, ensuring a good secondary response. In contrast, a good primary IgG response in itself may or may not guarantee a good secondary response (*i.e.* no memory cells produced). One might speculate that B cells secreting IgG in a primary response do not differentiate into memory B cells, and that sIgM bearing B cells seem to be a major population involved in primary IgM production and memory generation.

On the other hand, the present experiments may suggest that sIgM-CR2 co-expressing B cells preferentially become anergic

when treated with anti-CR2 prior to Ag stimulation, and therefore memory B cells will be lacking. However, as shown above (Fig. 6.23) once immunological memory is established, treatment with anti-CR2 does not induce suppression of a secondary response. This has also been found in the study of Thyphronitis et al. (1991).

7.6.3. Augmentation of secondary antibody response by Ag complexes through CR2

One of the numerous differences between the primary response and secondary response is the lower requirement for antigen in the secondary response. A second difference between the two responses may be that different populations of antigen processing/presenting cells are involved. Although macrophages play a major role in antigen handling (this may be considered the "classical antigen pathway"), B cells are not only capable of presenting antigen to T cells but also do so more effectively, at least in the secondary response.

The immune system consists of many highly organized microenvironments, one of which is the germinal centre (GC), located within follicles of peripheral lymphoid organs such as lymph nodes and spleen (Tew et al., 1990; Schriever and

Nadler, 1992; Kosco and Gray, 1992). Germinal centres develop transiently in response to an antigenic challenge, and through this development, antigen-specific memory B cell clones are generated, and switching of the immunoglobulin isotype and selection of clones with high affinity due to somatic mutations occur. Germinal centres contain a unique type of accessory cell, the follicular dendritic cell (FDC). The principal feature of all FDC is their ability to trap antigen in the form of immune complexes on their cell surfaces. Beaded "bodies of immune complexes" on the FDC surface were discovered by electron microscopy (Szakal et al., 1988) and are often referred to as "iccosomes". In this form, antigen could be taken up by GC B cells from FDC and presented to helper T cells, and the resulting T-B cell interaction leads to B cell proliferation. These steps have been proposed as the "alternative" antigen pathway (Tew et al., 1989), which includes: immune complex formation, immune complex trapping and transportation to FDC by non-phagocytic cells with dendritic morphology, iccosome formation, endocytosis of iccosomes by B cells, antigen processing and presentation of antigen to T cells.

There is a growing body of literature to support the concept that C3 is likely to be involved in the induction and

maintenance of memory B cells. Initial evidence came from the observation that mice depleted of C3 by CVF cannot localize Ag-Ab complexes on FDC (Klaus and Humphrey, 1977; Klaus et al., 1980).

In this study, using a mAb to CR2 instead of C3d fragments, we have established an animal model in mice for evaluation of the effect of CR2 on the secondary antibody response. To do this, all mice were first immunized with Ag to elicit a normal primary response. Ag complexed with mAb to CR2 was then used to test whether direct targeting of Ag to B cells or FDC via CR2 ligation can augment the secondary antibody response. We assume here that in the form of an immune complex with anti-CR2, antigens are likely to be handled in the same way as Ag-Ab-C3d complexes. The results demonstrate that the secondary antibody response can be triggered with 6.25 ug of free Ag [(goat F(ab')₂ in this case)] in primed mice. When less than this amount of Ag is used, the secondary antibody responses are barely detectable. However, when free Ag is complexed to anti-CR2 mAb, a 100-fold lower amount of antigen is capable of inducing the secondary antibody response. The enhanced antibody response was mainly due to the increased production of IgG_{2a} and IgG_{2b} subclasses.

One possible explanation for these observations is that administration of the preformed complexes may bind to Fc receptors and/or activate host complement. C3 fragments generated during activation may indirectly modulate the immune response. If this occurred the effect should be the same for both the anti-CR2 complex and anti-B220 complex. However, when animals were challenged by Ag complexed with anti-B220, the amounts of Ag needed to induce the secondary response were similar to those needed when Ag was injected alone. Nor are there reasons to believe that this occurs because of "loss" of Ag from anti-B220 complexes due to preferential binding of the IgG Fc components of anti-B220 to Fc_γ receptors on phagocytic cells (neutrophils). Anti-CR2 (7G6) and anti-B220 (RA3-6B2) are of the same isotype so a similar "loss" of Ag from anti-CR2 complexes should have occurred.

A second possible explanation is that anti-CR2 mAb in the complexed Ag may mediate antigen binding directly to the B cell. But, like anti-CR2, the control mAb against B220 should also target Ag to the B cells. Different results may suggest that anti-CR2 has a specific effect, either by signalling via CR2 on the B cells or by localizing effectively the Ag complexes on the FDC. Some possible interactions to explain the results of these experiments are summarized in Fig. 7.3.

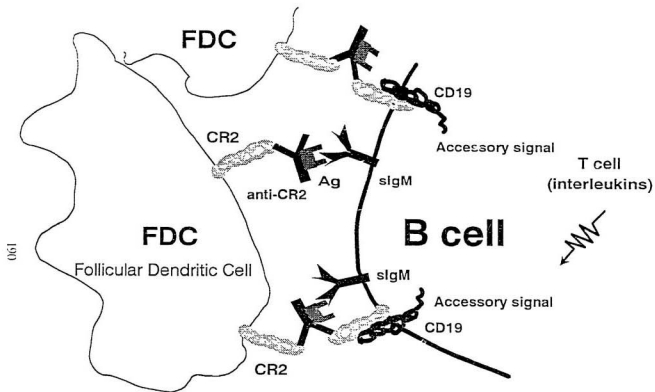


Figure 7. 3 Possible routes to B cell stimulation in the GC by Ag complexed with anti-CR2 (Y).

The mechanism by which anti-CR2 exerts immunoregulation of the secondary antibody response might involve the CR2-associated molecules on B cells and/or FDC since the 34 amino-acid cytoplasmic portion of CR2 itself appears to be too short to mediate transduction signals (Moore et al., 1987; Weis et al., 1988). In humans, one of these molecules is CD19, a member of the Immunoglobulin (Ig) superfamily (Zhou et al., 1991, 1992). Like CR2, CD19 is expressed on B cells throughout all stages except the plasma cell stage. CD19 has a long cytoplasmic tail with the capacity for signal transduction. The involvement of CD19 in B cell activation and proliferation has been documented in earlier studies (Carter et al., 1991). Recent studies have demonstrated that the CD19 molecule forms a multimolecular complex with CR2 and three other molecules (Matsumoto et al., 1991). Therefore, it has been proposed that the CD19 molecule serves as a signal transducing element of CR2. One set of in vitro experiments has shown that in the presence of saturating concentrations of mAb to CD19, the threshold for B cell activation was lowered and the magnitude of B cell proliferation was heightened when stimulated by mAb to sIgM (Carter and Fearon, 1992). The present data are consistent with these observations. In addition, the finding of a physical association between sIgM and CR2 in humans (Tuveson et al., 1991) may provide another mechanism by which

complement-coated antigens can stimulate B cells through crosslinking of both surface molecules. Association between CR2 and sIgM may occur in mice just as it does in humans. Obviously, these two possible mechanisms (CR2-CD19 and CR2-sIgM) are not mutually exclusive.

In addition, the experimental results could be explained by effective localization, via CR2, of Ag complexes on FDC (Sellheyer et al., 1989; Schriever et al., 1989). FDC express high levels of CR1 and CR2, and the function of the FDC here is probably to display the Ag complex so that antigen-specific B cells can easily react with the antigen. These B cells will be stimulated in the presence of T helper cells. This would be an efficient mechanism for generating large numbers of antigen-specific plasma cells in a short period of time. Under natural circumstances Ag-Ab-C3d immune complexes in vivo probably function in this way. Hence, a subtle immune response can be magnified, with the ultimate goal of eliminating foreign antigen. Of course, our data do not totally exclude the possible involvement of CR1, but in mice CR1 and CR2 are products of the same gene.

Because of both the complexity of immune response, the complexity of the complement system and the different, and

often incomparable experimental conditions used in different laboratories, some discrepancies between our data and the data of others are difficult to elucidate. For instance, sheep erythrocytes (SRBC), keyhole limpet hemocyanin (KLH) or bacterial antigen (*Salmonella*) had been used as Ag (Heyman et al., 1990; Thyphronitis et al., 1991). Future experiments should be carried out on transgenic mice made CR2 deficient with the knock-out CR2 gene.

7.6.4. Summary

Immunoregulatory functions of CR2 were explored in vivo in an animal model. Pretreatment of mice with anti-CR2 mAb significantly suppressed the primary antibody response (mainly IgM) to a T-dependent Ag, and impaired the generation of immunological memory. It is hypothesized that these effects might be due to the induction of B cell anergy. This suppression was different from that initiated by cobra venom factor (CVF) pretreatment. Treatment with anti-CR2 was unable to eradicate an already established immunological memory. The data also suggested that signalling via CR2 may preferentially affect switching from IgM to IgG₂. Finally, Ag complexed with anti-CR2, a physiological analogue of C3d-Ag-Ab, can evoke an in vivo secondary antibody response at a 100-fold lower Ag

dose than Ag alone, suggesting that anti-CR2 has a specific effect, either by signalling via CR2 on the B cells or by effectively localizing the Ag complexes on the FDC.

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